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(54) Title: HYBRID SEED PRODUCTION

(57) Abstract

Male sterility is imparted to a plant by a cascade of gene sequences which expresses a protein which disrupts the biosynthesis of viable pollen. Expression of the disrupter protein is restricted to male parts of the plant by an upstream promoter sequence which is specific to male flowers, the male specific promoter being under control of an operator sequence. The cascade also includes a gene encoding a repressor protein specific for that operator. Expression of the repressor protein is under control of a chemically inducible promoter which is inducible by the application to the plant by, spraying or like process, of an exogenous chemical. In the absence of the exogenous chemical inducer, no repressor protein is expressed, resulting in expression of the disrupter protein and, consequently, male sterility. Fertility may be restored to the plant, when required for maintenance of the line, by spraying with the inducer, resulting in expression of the repressor which binds the operator and inhibits expression of the disrupter protein.

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HYBRID SEED PRODUCTION

This invention relates to a method for the
production of hybrid plants. More particularly,
the invention relates to the molecular control of
fertility in crop plants.

5   Agriculture uses many crop plants for the
production of food for human consumption, for
commercial processes yielding products for human
consumption, for animal feedstuff production, for
the development of industrial products and other

10   purposes. The process invariably involves the
planting by the farmer of seed which has been
purchased from a seed producer. The product
produced by the crop be it the whole plant, the
seed or fruit of the plant, is harvested and is

15   then used for the various food applications
mentioned above. In addition to purchasing seed
from a seed company, the farmer also may plant back
some seed saved from a previous year's crop. This,
however, is only economically useful for crops

20   which are used as inbreds or outcrossed seed. It is
not economically advantageous for hybrid crops,
which are planted to realise the increase
productivity achieved by heterosis. The major
crops planted in the major agricultural regions are

25   planted as hybrid crops, which guarantee the farmer
considerable yield increases.

Production of hybrid seed by specialised seed
companies is a costly and complicated process. The
process involves the breeding and selection of
inbred parental lines, which in suitable
5   combinations give progeny which exhibit maximal
adaptation to environmental conditions with
realisation of maximal yield. For hybrid seed
production inbreds are chosen as either male or
female parent in a cross yielding F1 hybrid seed.
10   As most crops are hermaphroditic, the female parent
in a cross has to be emasculated in order to avoid
self-pollination during seed production. For open
pollinating plants like maize a special planting
regime is used in order to minimise self-
15   pollinating of the female parent. This involves
the separation of those plants to be used as males
from the female parents. This allows easy
separation of F1 hybrid seed at the end of the
season.

20   Emasculation can be achieved either
mechanically as it is used for maize, manually as
it is used for tomato, or chemically as it is used
for wheat or rice, and genetically using
cytoplasmic male sterility (CMS) or genetic
25   incompatibility as it is used for oil seed rape,
sugar beet and others. These approaches vary in
their complexity of agricultural practices or plant
manipulation, but have in common, that they are
costly to administer, complex to carry out and
30   relatively inefficient depending on which system is
used. This inefficiency arises from the fact that
fertile plants need to be sterilised for seed
production, which takes place in very large
acreages depending on the crop. Therefore, large

scale manipulation of females need to be carried out. Treatments also need to yield seed which is viable in the next gen ration which the farm r plants.

5   An object of the present invention is to obviate or mitigate the aforesaid disadvantages.

       According to the present invention there is provided a plant gene construct which includes a disrupter protein gene capable of disrupting the

10 biogenesis of viable pollen, and a gene control sequence which includes a promoter sequence inducible by external application of an exogenous chemical inducer to a plant containing the construct.

15   Preferably, the control sequence includes an operator controlling said disrupter protein gene and a repressor gene encoding a repressor protein adapted to bind said disrupter protein gene operator, said repressor protein gene being under

20 control of said chemically inducible promoter.

       Preferably also the construct includes a male flower specific operator sequence operatively linked to said disrupter protein gene, for restricting expression of the disrupter protein

25 gene to male flower parts of a plant.

       The invention also provides transformed plants and plant parts such as cells, protoplasts and seeds incorporating the construct of the invention.

30   In a preferred embodiment of the invention there is provided a recombinant DNA construct for insertion into the genome of a plant to impart restorable male sterility thereto, comprising:

(a) a first gene promoter sequence responsive to

the presence or absence of an exogenous chemical inducer,

(b) a gene encoding a repressor protein under control of the said first promoter sequence;

5 (c) an operator sequence responsive to the said repressor protein;

(d) a second gene promoter sequence expressible only in male parts of a plant; and,

(e) a gene encoding a protein inhibitor of a plant

10 characteristic essential to the production of viable pollen;

whereby the presence or absence of the exogenous chemical inducer enables selection of male fertility or sterility.

15 Further according to the invention there is provided a plant which is restorably male sterile, in which said plant contains, stably incorporated in its genome, the recombinant DNA construct defined above.

20 It is preferred that the said first promoter promotes expression of the repressor protein in response to stimulation by the exogenous chemical inducer whereby in the absence of the chemical inducer no repressor protein is expressed to

25 interact with the operator thus permitting expression of the gene encoding the inhibitor of male fertility and in the presence of the chemical inducer repressor protein is expressed thereby preventing expression of the gene encoding the

30 inhibitor of male fertility and restoring the plant to the fertile state. Thus the construct of the invention contains several operatively linked sequences (a) above will be referred to for convenience as "the chemical switch": (b) as "the

repressor sequence" (c) as "the operator" (d) as
"the MFS control" (i.e male flower specific
control) and (e) as the disrupter gene. The
essential elements of each of the sequences and
their interaction will be described below with
reference to the accompanying drawings.

This invention enables the production of
inbreds which are rendered male sterile using
various molecular techniques and approaches. Thes
plants require a chemical switch system for the
reversal of fertility (reversible male sterility;
RMS). Both aspects of the system are inherited as
Mendelian characters. This will be achieved
through the insertion into the plant genome of the
molecular elements required for the controlled
function of RMS.

The invention can be used for any mono- or
di- cotyledonous plant which the breeder or grower
wants to produce as F1 hybrid seed and for which
suitable transformation techniques are or become
available, particularly maize, wheat, sunflower,
oil seed rape, tomato and other vegetables, sugar
beet and ornamental foliage and flowering plants.
It has great advantages in reduction in crop
management costs associated with F1 hybrid seed
production, ease of purity control of hybrid seed,
maintenance of RMS lines and populations, transfer
of RMS between lines and populations etc.

In one specific application we shall describe
the production of plants, particularly inbred
plants, which are rendered sterile using molecular
engineering approaches. These plants can be
reversed to fertility using a chemical spray which
leads to the restoration of fertility using a

molecular control cascade. The method presented
here consists of a number of individual components
which are subject to separate patent applications
which disclose wider applications of the
5   components.

1.   THE OVERALL PROCESS

        Figure 1 of the drawings is a block diagram of
the DNA construct of the invention in the "male
sterile" state. In the absence of the exogenous
10   chemical inducer, the chemical switch is inactive
and no repressor protein is expressed by the
repressor sequence. In the absence of the
repressor protein, the operator sequence permits
expression of the disrupter protein in male
15   specific tissue, expression being specifically
directed to male parts of the plant by the presence
of the MFS control sequence. The outcome being
that the plant is male sterile, is unable to
produce viable pollen and thus unable to
20   self-pollinate. The practical utility of this
effect is that if such plants, representing the
female of an intended cross, are planted proximate
the intended male parent of the cross, it will be
pollinated by pollen from the intended male.

25   Figure 2 shows the operation of the construct
in the "male fertile" state. When the chemical
inducer is brought into contact with the plant, the
chemical switch is activated causing the repressor
protein to be expressed. The repressor protein
30   then binds the operator, inhibiting expression of
the disrupter protein and restoring male fertility.
The practical utility of this mode of operation of
the construct is that it allows production of
viable pollen and self- pollination of the plant

containing the construct in order that the line may be perpetuated for future use.

2. THE CHEMICAL SWITCH

A large number of plant promoters are assumed to be induced using chemical signals. However, it has only been demonstrated in few examples that the specific chemicals switch on gene expression in the tissue required for this model. The gene of particular interest is the gene encoding the 27k subunit of glutathione-S-transferase II (GSTII). This gene is induced specifically upon treatment of plant tissues, particularly developing anthers using chemical safeners. One such safener is N,N-diallyl-2,2- dichloroacetamide, but there are related compounds which have improved mobility characteristics in plants tissues, combined with improved persistence for this application, efficacy and safety. These compounds have been described in the literature.

It is obvious that additional chemically induced promoters can be used in this scenario. Some of these may be of plant origin, others may be of fungal or yeast origin. It is implied in the present application that those promoters and chemical combinations suitable for the RMS procedure can be used in case of GSTII and safeners.

3. THE REPRESSOR AND OPERATOR SEQUENCES

In a first embodiment we propose to use the well- characterised interaction between bacterial operators with their repressors to control the expression of the 'killer' gene function. Bacterial repressors, particularly the lac repressor, or repressors used by 434, P22 and lambda

bacteriophages can be used to control the
expression in plant cells very effectively.

In a second embodiment it is possible to
utilise 'pseudo-operators', operators which are
5   similar but not identical to the normally used
operators in a particular operator-repressor
combination. We have demonstrated that using a
suitable selection system mutant repressors can be
generated which recognise pseudo-operators found in
10   plants genes. We describe below the selection of
mutant repressors recognising pseudo-operators
which are found in plant genes.

Another approach for the down regulation of
the 'killer' genes which can be considered is the
15   use of antisense RNA. This has been demonstrated to
work well for the regulation of polygalacturonase
expressed during tomato fruit ripening and is
described in the literature.

4. MALE FLOWER SPECIFIC EXPRESSION CASSETTE

20   As already mentioned, the expression of the
'killer' genes has to take place in male flower
specific tissues (MFS). These can be tissues found
in the developing anthers, in tissues associated
with the developing anthers and pollen.

25   DNA promoter sequences which drive the
expression of genes in MFS tissues can be achieved
using established protocols for the identification
of genes expressed in MFS tissues through
differential screening of cDNA libraries cloned in
30   various vector systems, the isolation of genes
encoding these cDNAs from genomic libraries using
bacteriophage lambda vectors, and the
characterisation of their promoter sequences using

DNA sequencing and analytical plant transformation
experiments.

5. DISRUPTER GENE

Inhibition of pollen formation will be
achieved by using novel 'killer' genes which, when
expressed specifically in male flowers during
pollen formation (for details see above), will lead
to cell death of the anthers and associated
tissues, pollen mother cells, pollen and associated
tissues. This will lead to the abortion of pollen
formation, and plants which are male sterile. The
origin of the 'killer' genes can be from a variety
of naturally occurring sources, eg human cells,
yeast cells, plant cells, fungal cells, or they can
be totally synthetic genes which may be composed of
DNA sequences some of which are found in nature,
some of which are not normally found in nature or a
mixture of both. The 'killer' genes will have
preferably an effect on mitochondrial metabolism,
at it has been quite clearly demonstrated that
ample energy supply is an absolute requirement for
the production of fertile pollen. However, it is
also envisaged that the 'killer' function can be
effectively targeted to other essential biochemical
functions such as DNA metabolism, protein
synthesis, and other metabolic pathways. Two such
DNA constructs consist of those sequences encoding
the mammalian brown adipose tissue uncoupling
protein or variants thereof, or a synthetic gene
which consists of a mitochondrial targeting domain,
and a lipophilic domain which allows insertion of
the protein into the mitochondrial membrane.

6.   PRODUCTION OF AN EXPRESSION MODULE CONSISTING
      OF MFS PROMOTER SEQUENCES AND 'KILLER' GENES

Production of an expression module which consists of the male flower specific gene control sequences (MFS control) and the 'killer' genes will be done using established molecular techniques. The expression of this module in elite inbreds will lead to the production of the 'killer' gene product only in male flower specific tissues. This will lead to the production of male sterile plants.

7. TRANSFORMATION

Transgenic plants are obtained by insertion of the constructs described into the genome of the plants. The specific transformation procedure employed for insertion of the gene constructs of this invention into the plant genome is not particularly germane to this invention. Numerous procedures are known from the literature such as agroinfection using Agrobacterium tumefaciens or its Ti plasmid, electroporation, microinjection of plant cells and protoplasts, microprojectile transformation and pollen tube transformation, to mention but a few. Reference may be made to the literature for full details of the known methods.

8. REVERSAL OF STERILITY

It is apparent, that plants which are made sterile using the above techniques and methods are not desirable per se. Therefore we proposed to use a cascade using molecular elements which will allow the reversal of the engineered sterility to fertility thus permitting maintenance of these plants and their use in F1 hybrid seed production.

Design of the reversal mechanism

The reversal mechanism proposed here consists of three separate elements:

a. a chemically switchable promoter

b. a bacterial operator sequence

c. a bacterial repressor gene which binds with high affinity the aforementioned operator.

These elements will act in the following way:
when restoration of fertility is required (eg in
the inbred maintenance plots), plants are sprayed
with a chemical. This chemical induces through a
chemically- inducible promoter the expression of a
bacterial repressor molecule which will bind to
operator DNA sequences in the MFS control
sequences. This binding will lead to the inhibition
of the 'killer' gene function, thus allowing normal
pollen formation to take place.

9A. APPLICATION TO F1 HYBRID PRODUCTION

Figure 1 outlines the molecular events which
will take place when RMS is used in elite plants.
Three genetic scenarios are available for the
expression of the introduced traits, depending on
whether they are destined to act as dominant or
recessive genes and on whether expression is
arranged to occur in the tissues of the parent
sporophyte or gametophyte. These scenarios are
summarised in Table I below.

TABLE I
PRODUCTION OF F1 HYBRIDS

Genetic Status of Expression	RMS Genotype			F1 fertile Pollen Load
	Female (MS)Inbred	Male Inbred	F1 Hybrid	
Sporophytic dominant	RMS/+	+/+	RMS/+ or +/+	50% plants (+/+) are 100% fertile
Sporophytic recessive	rms/rms	+/+	rms/+	100% plants are 100% fertile
Gametophytic	RMS or RMS	+ or +	RMS + or +	50% plants are 50% fertile

Where, for example, the RMS components, particularly the "killer gene" functions are expressed as dominant gene(s), it is advisable to utilise RMS in a heterozygous state (RMS/+ in the above-mentioned "Sporophytic dominant" classification). It is implied in this instance that during F1 hybrid seed production, the resulting hybrids are either RMS/+ or +/+ in their genotype. This means that only 50% of the plants (+/+) will be able to contribute to pollen production in the F1 used for grain production. This will be sufficient for most crops,

particularly in maize in which a large amount of pollen is produced.

Importantly, RMS can also be used in the production of other classes of hybrids such as three-way and four-way crosses. In the above-mentioned embodiment using the dominant sporophytic expression, use of the female genotype RMS/RMS in the first cross conveniently generates the genotype RMS/+ on crossing with a +/+ pollinator. The rms/+ genotype then constitutes the male sterile female parent for use in the second cross.(See Table II below).

TABLE II
PRODUCTION OF 3 AND 4 WAY CROSSES

Genetic Status of Expression	RMS Genotype				
	First Female	First Male	Second Female	Second Male**	3 or 4 way Hybrid
Sporophytic Dominant	RMS/RMS	+/+ ➞	RMS/+	+/+	RMS/+ or +/+
Sporophytic Recessive	rms/rms	ms/rms* ➞	rms/rms	+/+	rms/+
Gametophytic	RMS or RMS	RMS or RMS* ➞	RMS or RMS	+ or +	RMS or +

* In these embodiments the first pollinator is treated with the chemical switch to restore male fertility.
** The second pollinator may be an inbred (3 way) or or a hybrid (4 way)

9B. APPLICATIONS IN BREEDING PROGRAMMES

In addition to its broad utility in hybrid
seed production, the RMS invention is also
conveniently utilised in breeding programmes, for
example, in enforcing outcrossing in synthetic
populations from which new inbred lines are to be
derived. Scenarios similar, but not limited to
those shown in the above Table for the first cycle
in production of three and four way crosses may be
employed. Use of the chemical switch facilities
subsequent rounds of selfing cycles to produce new
and improved genotypes Molecular screening (RFLP
analyses) using DNA sequences derived from the DNA
sequences used to facilitate the RMS invention may
be conveniently used as probes during inbreeding to
ensure that progeny to be advanced have retained
all elements of the RMS system. These strategies
offer a convenient and efficient procedure to
transfer the RMS trait to new germplasm.

The invention will now be described, by way
of illustration by the following specific
descriptions of various component gene modules
which may be utilised. Reference will be made to
the accompanying drawings.

THE DRAWINGS

Figure 1 is a block diagram of the gene
construct of this invention with the plants in the
male sterile state;

Figure 2 is a block diagram of the gene
construct of this invention with the plants in the
male fertile state;

Figure 3 shows the results for total GST
activity in roots and shoots obtained 23 and 44
hours after treatment with R25 as described below;

Figure 4 shows the chromatographic separation of the isozymes GST I and GST II.

Figure 5 shows GST I activity present in untreated anther tissue;

5   Figure 6 shows the stimulation of GST II activity after treatment with R25 as described below;

Figure 7 shows the results using a stem reservoir technique;

10   Figure 8 shows the results with the application by spray; and,

Figure 9 is a time course graph generated in the manner described below.

Figure 10 shows the structure of vector

15   p35SlacI.

Figure 11 shows the basic structure of pCG1 and pCG2;

Figure 12 shows the nucleotide sequence of the maize CAB promoter;

20   Figure 13 is a map of the pAD and pPS1 series of plasmids;

Figure 14 shows the library screening procedure used for the isolation of maize flower specific clones;

25   Figure 15 shows dot blot analysis of total RNA (4μg per dot) extracted from maize tassels of increasing length.

Figure 16 A, B, C shows in situ hybridisation of maize spikelet sections with pMS14 antisense RNA

30   probes.

Figure 17 shows the nucleotide and deduced amino acid sequence of MFS cDNA clone pMS10;

Figure 18 shows the nucleotide and deduced amino acid sequence of MFS cDNA clone pMS14;

Figure 19 shows the nucleotide and deduced
amino acid sequence of MFS cDNA clone pMS18;

Figure 20 is a restriction map of the 9kb
EcoRI fragment from clone 10/CT8-3;

5   Figure 21 is a restriction map of the 9kb
EcoRI fragment from clone 14/17M;

Figure 22 is a restriction map of the 9kb
EcoRI fragment from clone 18/CT3;

Figure 23 is a plasmid map of clone pMS10-5;

10   Figure 24 shows the structure of pTAK1, pTAK2
and pTAK3; and,

Figure 25 is a map of clone pMS10-6GUS.

Figure 26 is a map of plasmid pCGS110-UCP;

Figure 27 shows the mRNA sequence of mammalian
15   uncoupling protein gene from plasmid pCGS110-UCP
(shown in Figure 24);

Figure 28 is a flowchart representation of the
generation of a leu2, gal1 yeast strain;

Figure 29 is a table showing the effect of
20   addition of galactose on the growth of BET9 and
BET27 transformants;

Figure 30 shows results of growth curve
analysis of BET9 (Figure 28A) and BET27 (Figure
28B) transformants grown on gly/cas medium over a
25   period of 65 hours in the presence or absence of
galactose;

Figure 31 is the growth curve analysis of rat
UCP in strain BET9 grown on gly/cas medium over a
period of 50 hours in the presence or absence of
30   galactose;

Figure 32 is the growth curve analysis of rat
UCP in strain BET9 grown on raffinose medium over a
period of 45 hours in the presence or absence of
galactose;

Figure 33 illustrates the construction of
plasmid YIP/UCP from pKV49-UCP and YIp5;

Figure 34 is a map of plasmid pGR208 (Figure
32A) and the sequence of oligonucleotides used to
5   mutate the β-subunit gene of F_1-ATPase (Figure
22B);

Figure 35 shows maps of plasmids pKV49 (Figure
33A) and pKV49-UCP (Figure 33B);

Figure 36 shows schematically the construction
10   of a β-subunit/β-galactosidase fusion protein;

Figure 37 is a plasmid map of pKV49/BLZ;

Figure 38 is a plasmid map of pMS10-5; and,

Figure 39 is a plasmid map of pBin/MS10-UCP.

THE PLASMIDS

15   Various gene constructs are described in the
following paragraphs and these have been deposited
in the National Collection of Industrial & Marine
Bacteria in Aberdeen, UK. The dates of Deposition
and the Accession Numbers are summarised in Table
20   III below.

TABLE III

Plasmid	Host	Deposit Date	Accession No.
pGAL2	E.coli DH5α	06 Dec 1988	NCIB 40087
p35SlacI	E.coli TG-2	12 Dec 1988	NCIB 40092
pPS1	E.coli DH5α	21 Dec 1988	NCIB 40097
pAD18	E.coli DH5α	21 Dec 1988	NCIB 40096
pMS10	E.coli RR1	09 Jan 1989	NCIB 40098
pMS14	E.coli DH5α	09 Jan 1989	NCIB 40099
pMS18	E.coli RR1	09 Jan 1989	NCIB 40100

I. THE CHEMICAL SWITCH

This module is exemplified by a chemically inducible gene promoter sequence isolated from a 27kd subunit of the maize glutathione-S-transferase (GST II) gene.

In practice the chemically inducible promoter of the invention will be inserted as a promoter sequence in a recombinant gene construct destined for use in a plant. The construct will then be inserted into the plant by transformation. Expression of protein encoding genes in the construct, being under control of the chemically switchable promoter of the invention, may be controlled by the application of a chemical inducer to the plant.

Examples of effective promoter/inducer combinations are the promoter is the GST II promoter aforesaid and the inducer is N,N-diallyl-2,2- dichloroacetamide (common name: dichloramid) or benzyl-2-chloro-4-(trifluoromethyl) -5-thiazole- carboxylate (common name: flurazole)

Chemical inducers which are potential inducers of the GSTII 27kd subunit expression include compounds such as:

1. benzyl-2-chloro-4-(trifluoromethyl)-5-thiazole-carboxylate;

2. naphthalene-1,8-dicarboxylic anhydride;

3. 2-dichloromethyl-2-methyl-1,3-dioxolane;

4. 1-(dichloroacetyl)-hexahydro-3,3,8a-trimethyl-pyrrole (1,2-a)-pyrimidin-6(2H)-one;

5. 2,2,5-trimethyl-N-dichloroacetyloxazolidine;

6. 1,3-dioxolan-2-ylmethoxyimono(phenyl)benzene acetonitrile;

7. 4,6-dichloro-2-phenyl-pyrimidine;

8.   2,2-dichloro-[N-allyl-N(1,3-dioxalano-2-
     methy)] acetamide;

9.   1-(cyanomethoxyimino)benzacetonitrile;

10. 4'-chloro-2,2,2-trifluoroacetophenone-O-1,3-
     dioxolan- 2-yl methyloxime;

11. 2,2-dichloro-1-(3,4-dihydro-3-methyl-2H-1,4-
     benzoxazin-4-yl) ethanone;

12. 3-dichloroacetyl-2,2-dimethyloxazolidine;

13. 4-methoxy-3,3-dimethylbenzophenone;

14. 1-cyclohexyl-4,4-dimethyl-2-(1H-1,2,4-
     triazol-1-yl) pent-1-en-3-ol;

15. 2,2-dichloro-N-(3-methyl-4-thiazolin-
     2-ylidene) acetamide;

16. O,O-diethyl-O-phenyl phosphorothioate;

17. 2,2-spirocyclohexyl-N-dichloroacetyl
     oxazolidine;

18. N-benzyl-N-ethyl-dichloroacetamide;

19. 3-chloroacetyl-4,4-cyclohexane-spiro-2,2-
     dimethyl-1,3- oxazolidine; and,

20. spirooxazolidine acetamide.

     Glutathione-S-transferases (GST) are a family
of enzymes which catalyse the conjugation of
glutathione via the sulphydryl group to a large
range of hydrophobic, electrophilic compounds. The
conjugation results in detoxification of these
compounds and in insects and mammals, removal from
tissue.

     GST enzymes have been identified in a range of
crop plants including maize, wheat, sorghum and
peas. GST's comprise from 1 to 2% of the total
soluble protein in etiolated maize seedlings.

     The major isoform of GST can be distinguished
in maize tissue. GST I is constitutively expressed
and is capable of conjugating glutathione with the

pre-emergent herbicides alachlor and atrazine.
Treatment of maize tissues with chemical safeners
(for example, N,N-diallyl-2,2-dichloroacetamide)
raises the activity of GST I which participates in
5   the detoxification of the pre-emergent herbicides.
Safener treatment of corn tissue

For treatment of young maize seedlings, seeds
were germinated on moist filter paper. After
germination and growth (up to one week) the safener
10   N,N-diallyl-2,2- dichloroacetamide (hereinafter
referred to as R25) was added to the water in the
filter paper to give a range of concentrations
(0.003 to 30 ppm) and the seedlings grown for a
further 23 to 44 hours before harvesting of root
15   and shoot tissue. Figure 3 shows the results for
total GST activity in roots and shoots obtained 23
and 44 hours after treatment as described and
Figure 4 shows the separation of the isozymes GST I
and GST II.

20   For treatment of maize tassel and anther
tissue, a solution of 800 μg of R25 was injected
into the node directly below the developing tassel.
Uptake then continued for a further 48 to 72 hours.
Figure 5 shows that only GST I activity was present
25   in untreated anther tissue and Figure 6 shows the
stimulation of GST II activity after treatment as
described.

Alternatively a 100 ppm solution of R25 was
supplied from a glass reservoir attached to the
30   exposed stem immediately below the developing
tassel. Figure 7 shows the results using a stem
reservoir technique.

Additionally, R25 was applied as a 100 ppm
spray directly on to the exposed developing tassel.

Figure 8 shows the results with the application by spray.

Both GST proteins have a native molecular weight of approximately 50 kd. As in mammals, maize GST's are dimeric; GST I has apparently identical subunits of 29 kd, whereas GST II is a heterodimer of a 29 kd subunit similar to that found in GST I and a novel 27 kd subunit which is only present in tissue treated with safener except in seedling root where it is constitutively expressed, but still can be induced by safener treatment.

A cDNA and a gene corresponding to the 29 kd subunit of GST I have been cloned previously and sequenced. In addition, a cDNA corresponding to a 26 kd subunit of a third, minor component of GST activity in maize seedlings (GST III) has been previously cloned and sequenced.

Enzyme Assay

Enzyme activity was measured spectrophotometrically at 340nm using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The reaction buffer contained 0.1M EDTA, 0.001M CDNB and 0.0025M glutathione.

Preparation of extracts and enzyme purification

Tissue was homogenised in 0.05M Tris.HCl, pH 7.8; 0.001M EDTA; 0.001M DTT; and 7.5% polyvinylpyrrolidone in a pestle and mortar, at 4°C, and centrifuged at 30,000g to obtain a crude extract.

Separation of the GST isoforms from the crude extract was achieved as follows: the crude extract was applied to a DEAE Sepharose column and washed

with 0.01M Tris.HCl, pH 7.8; 0.001M EDTA; and
0.001M DTT. The bound GST was eluted with 0.3M
potassium chloride. Fractions containing GST
activity were combined and desalted using PD10 gel
5   filtration columns. Separation of the GST I and
GST II isoforms was achieved by FPLC on a mono-Q
column and a zero to 0.4M potassium chloride
concentration gradient.

Pure samples of GST I and GST II were obtained
10   by applying desalted fractions of GST I and GST II
from the FPLC to a glutathione-S-sepharose affinity
column equilibrated with 0.05M phosphate buffer at
pH 7.3. After washing with buffer, bound GST was
eluted with 0.005M glutathione.

15   SDS-PAGE (17.5%, 30:0.174 acrylamide:
bisacrylamide) of GST I or GST II was achived by
concentrating pure GST samples using Amicon
Centricon 10 Microconcentrations (Trade Mark),
denaturing samples in mercaptoethanol containing
20   Laemmli buffer, and staining the gels with
Coomassie Blue.

Generation of antibodies to the enzyme

Sufficient protein to enable the immunisation
of rabbits is obtained by pooling the isolated
25   enzyme subunit isolated as described above from a
number of separate experiments. The 27 kD GST II
polypeptide is subsequently purified to apparent
homogeneity by electroelution from polyacrylamide
gel slices. Antisera are prepared against the 27 kd
30   polypeptide. The immunisation of rabbits is carried
out essentially according to Mayer and Walker
(1978).

N-terminal sequence analysis

    The amino terminal sequence of the intact 27
kd subunit of GST II or partial proteolytic
cleavage products was determined by sequential
5   Edman degradation and subsequent amino acid
analysis by HPLC.

Time Course

    A time course experiment was carried out to
examine the expression of GST's after safener
10   treatment. A 30 ppm solution of R25 was applied to
three-day old seedling roots and tissue harvested
after various time intervals following safener
treatment. Samples were tested for GST activity
using the enzyme assay described above. The
15   results of this experiment are presented
graphically in Figure 9.

Synthesis of cDNA libraries

    The time course experiments revealed a peak of
GST expression at 48 hours after treatement with
20   safener. Therefore, two cDNA libraries were
constructed from RNA extracted from tissue at 24
and 48 hours after safener treatment. To ensure
that the induction procedure had been successful, a
one gram sample of 24 hour induced tissue was taken
25   and assayed for GST II. This experiment revealed
that the tissue used to construct the cDNA library
had indeed been successfuly induced as GST II
accounted for 45.5% of the total GST activity.

    Double-stranded cDNA was prepared from oligo
30   dT-cellulose-purified RNA by a method employing
RNaseH and E.coli DNA polymerase I in the synthesis
of the second strand, without prior purification of
single-stranded cDNA (Gubler and Hoffman,1983).

Screening cDNA libraries with antisera to GST I and GST II

In order to identify a cDNA clone encoding maize tassel GST enzyme, bacteriophage from the amplified cDNA library are screened with anti-maize GST enzyme serum. The clones producing the strongest signals are re-screened.

Screening cDNA libraries using oligo probes

Mixtures of synthetic oligonucleotides based on the amino acid sequence determined above were prepared by phosphoramidite chemical synthesis. The 5' ends of the oligonucleotides were labelled using polynucleotide kinase as described in the literature.

Approximately 40,000 phages containing cDNA were amplified on plates and transferred to nitrocellulose. The filters were hybridised to oligonucleotide probes at temperatures of from 2 to 5°C below the melting temperature calculated for the lowest melting point probe in the mixture. Hybridising plaques were selected and rescreened through two or more rounds exactly as described above but at lower densities

Isolation of cDNA gene sequences by the PCR method

cDNA or DNA sequences are isolated from the libraries described using oligo primers based on the amino acid sequence obtained from partial proteolytic cleavage or in the case of genomic DNA, primers based on cDNA sequence determined previously.

Characterisation and sequence analysis of GST cDNA clones

The isolated cDNA is characterised and subjected to sequencing by one or more of the

standard available techniques.

Isolation of genomic sequences

An existing genomic library of fragments of
total maize DNA cloned into λEMBL3 is used to
5   isolate clones that hybridise to the cDNA clones
isolated as described above.

Alternatively, the PCR method described above
may be used to selectively amplify and clone gen
fragments. GSTII genes and ther promoter sequences
10 can then be isolated, and characterised using
established techniques. It can be demonstrated that
the GSTII promoter sequences mediate
safener-induced gene activity by fusing them to
marker genes like GUS and CAT, and testing then in
15 transgenic plants.

II. THE REPRESSOR AND OPERATOR SEQUENCES

This module regulates plant gene expression.
More particularly, the module regulates plant gene
expression by the use of repressor molecules of
20 bacterial or lower eukaryotic origin.

Traditionally, the improvement of crop
plant species involves the introduction of desired
traits by genetic crosses. However, although these
breeding techniques are highly successful, they
25 provide no means of controlling the expression of
the newly acquired traits. Recent advances in
technology are now allowing the genes responsible
for determining plant structure and the
productivity and quality of the crop to be
30 identified and isolated. A major aim in the field
of improvement is therefore to be able to
manipulate complex developmental processes
genetically in order to improve crop performance.
Essential to this objective is the determination of

strategies which allow the expression of specific plant genes to be regulated at will.

The ability to control the expression of traits according to circumstances has many
important applications, such as control of insect resistance genes, determination of plant height and timing of flowering and the control of plant fertility. Additionally, the ability to switch genes on or off at will, without disturbing plant physiology or environment, would be an invaluable tool in the study of plant genetics per se.

Currently, the production of seed for hybrid crops such as maize involves the laborious and expensive process of hand or mechanical emasculation of the parent plants in order to prevent self-pollination. Such emasculation can, however, be controlled genetically by making use of a trait known as cytoplasmic male sterility (CMS) which has been observed in a wide variety of crop species. CMS interferes with male gametogenesis, resulting in the inhibition of pollen formation, but does not normally affect female fertility. Consequently, "male-sterile" plants are able to set seed, such seed resulting only from cross-pollination. The ability to control the expression of these genes would allow male gametogenesis to be inhibited in the production of hybrid crop seeds without the need for expensive emasculation processes, while still allowing genetic improvement of the male parent by conventional breeding programmes.

Control of gene expression in both prokaryotes and eukaryotes relies primarily on the interaction of regulatory proteins with specific DNA sequences.

Depending on the nature of these interactions, transcription from the cognate promoters may either be repressed or activated. Indeed, in some cases the same protein may either reduce or enhance transcription according to the nature of the contacts made. Furthermore, the ability of some regulatory proteins to bind their target sequences is modulated by the binding of ligands or by specific proteolytic cleavage. Such mechanisms may be exploited in order to include inducibility amongst strategies for plant gene regulation.

The best characterised regulatory systems are those of bacteria in which the interactions between the DNA-binding proteins (repressors) and the target DNA sequences (operators) are understood in great detail. A comparison of the best understood systems, including repressor and cro proteins of bacteriophage λ and 434, the LacI repressor and the catabolite gene-activating protein (CAP), reveals several factors in common. These regulatory proteins bind as dimers or tetramers to short operators that exhibit a high degree of dyad symmetry. In most cases the domain responsible for DNA-recognition, which is separate from that concerned with oligomerisation of the monomers, contains a conserved helix-turn-helix structure. A specific helix within this structure in each monomer, the recognition helix, is aligned with the major groove of the DNA and only if specific contacts are formed between the amino acids of this recognition helix and the bases of the adjacent DNA can a functional repressor/operator complex be

formed. Such interactions are highly specific, and the high-affinity complexes are formed with extremely rapid kinetics.

The knowledge of mechanisms by which gene expression is regulated in eukaryotes is much less detailed. In yeast and mammalian cells a large number of binding sites for putative regulatory proteins have been identified in promoter sequences, and in some cases the proteins responsible have also been isolated. However, only in a few instances are the molecular details known of the protein-DNA interactions and the mechanism by which transcription is regulated.

In plants, regulation of gene expression is understood at only a rudimentary level. Several regulatory elements have been identified in promoter sequences, and some regulatory proteins examined at a preliminary level. However, such proteins have yet to be isolated and the details of the mechanisms involved elucidated.

Eukaryotic regulatory systems appear to exhibit a greater diversity of structure and a higher degree of complexity than their prokaryotic counterparts. For instance, control of transcription from eukaryotic promoters is thought to involve the interaction of many proteins (perhaps in the order of tens) with the regulatory DNA. Furthermore, at least three different protein structures (the helix-turn-helix, the zinc-finger and the leucine zipper) have been implicated in the specificity of DNA-binding by various eukaryotic regulatory factors.

DNA-binding proteins constitute a class of
proteins characterised by their ability to bind to
DNA of genes to give the effect of either
repressing or activating the gene to which they
5   bind. Unless the context otherwise requires, such
DNA-binding proteins will hereinafter be referred
to for convenience simply as "repressors".

This module, then, is a recombinant plant gene
comprising a repressor gene of bacterial origin and
10   a promoter which operates in plants for driving
expression of the repressor gene, said gene
encoding a repressor protein capable of interaction
with an operator sequence associated with a
selected target plant gene so that on expression of
15   the repressor protein expression of the target
plant gene is inhibited.

A vector, designated p35SlacI, containing the
said DNA, which has been deposited in an E.coli,
strain TG-2, host with the National Collection of
20   Industrial and Marine Bacteria Limited, Aberdeen,
United Kingdom, on 12th December 1988, under the
Accession Number NCIB 40092.

A suitable plant transformation vector
comprises Agrobacterium tumefaciens, harbouring the
25   plasmid aforesaid.

In a specific embodiment of this module a
bacterial lacI operator system is utilised to
regulate gene expression. Lac repression can be
relieved by iso-propyl thiogalactoside (IPTG) and
30   other sugar analogues.

Specific Examples relating to this module will
now be given.

EXAMPLE

(1) Construction of plants expressing
     the lac repressor

Vectors were constructed which express the
lacI gene from either the constitutive CaMV 35S
promoter found in vector pJR1 or from the green
tissue- specific promoter, the maize CAB promoter.
However, the bacterial repressor can be expressed
from any plant promoter expressed in other parts of
the plant, thus allowing control of plant gene
expression in any specific part of the plant.

(1.1) Modification and insertion
      of the lacI repressor gene into pJR1

The lac repressor (lacI^Q) is available on
plasmid pMJR 156. In order to express this gene in
plants, the translation initiation codon (GTG) had
to be changed into ATG. In addition it was
opportune to create a suitable restriction enzyme
cleavage site for cloning of this gene into a plant
expression vector. At the 3' end of the lacI there
are suitable restriction sites (HindIII and PstI)
for insertion into plant expression vectors. In
order to create suitable restriction sites at the
5' end, the following experiments had to be
performed:

(a) A Cfr 10 restriction site is located at
position 134. pMJR was cut with Cfr 10 and a
synthetic DNA fragment which reconstitutes the
N-terminus of the lacI gene, the altered
translational start codon ATG, a plant consensus
sequence for efficient translational initiation and
a BamHI restriction site were inserted into pJR1.
The sequence of this synthetic fragment was:

BamHI consensus

GATCC AACAATGGCT AAACCAGTAACGTTATACGATGTCGCAGAGTAT G

    G TTGTTACCGA TTTGGTCATTGCAATATGCTACAGCGTCTCATA CGGCC

                                                      Cfr 10

5   pJR1 was cut with BamHI and PstI. The
synthetic fragment described above, and the Cfr10 to
PstI fragment containing the lacI gene were ligated
together with the cut vector pJR1 under standard
conditions. The ligation mix was transformed into
10   E.coli TG-2. Recombinants were selected on
kanamycin-containing plates. They were
characterised by DNA sequence analysis. The
construct was designated p35SlacI.

(b) The PCR (Polymerase Chain Reaction) as
15   described by Saiki et.al., Science, 239, 487-491)
was utilised to introduce the changes at the 5' end
of the lacI gene while keeping the sequence at the
3' end. Two oligonucleotides were hybridised to
pMJR 156. The sequence of the oligonucleotides
20   were:

(i) from the 5' end of the gene

                       BamHI   consensus

GAGAGTCAATTCAGGGT GGATCC AACAATGGCT

AAACCAGTAACGTTATACG

25   (ii) from the 3' end of the gene

CGTTGTAAAACGACGGCCAGTGCC

       The PCR reaction was carried out under the
prescribed conditions. The product was cut with
BamHI (at the newly introduced site) and PstI. The
30   resulting fragment was cloned into pJR1 cut with
BamHI and PstI. Recombinants were identified
by hybridisation and restriction analysis using

standard protocols. One of the resulting clones was
characterised by DNA sequence analysis.

Both of the methods (a) and (b) gave the same
construct, designated p35SlacI. Figure 10 shows the
structure of vector p35SlacI.

(1.2) Replacement of the CaMV 35S promoter
    with maize CAB promoter

In order to demonstrate the general utility of
the Lac repressor/operator system in plants, we have
constructed an expression vector which will allow
inducible and tissue-specific lacI expression in
plants. For this work, we have used the promoter of
the gene encoding the light-inducible maize
chlorophyll a/b binding protein (CAB).

The construction of this vector was achieved
by replacing the CaMV promoter in p35SlacI with the
maize CAB promoter, the DNA sequence of which is
given in Figure 12 herewith, which is found in
vector pCAB48.1. The CaMV promoter was removed by
restriction of p35SlacI with EcoRI and BamHI using
standard conditions. The CAB promoter was isolated
from pCAB48.1 by restriction with XbaI and Sau3A
using partial restriction conditions for Sau3A.
This promoter fragment was then inserted into the
promoter-less p35SlacI. This vector, designated
pCABlacI, has been characterised by restriction
mapping and DNA sequence analysis.

(1.3) Transformation of tobacco plants

The expression modules from the vectors
described above were transferred to BIN19 and then
to tobacco using leaf disc transformation following
standard protocols. The plasmids were transferred
to _Agrobacterium_ using triparental mating.
Agrobacteria were purified, and used in leaf disc

transformation experiments. Thirty-seven plants
containing the CaMV-lacI expression module and
thirty-eight plants containing the BAB-lacI
construct were regenerated and analysed for the
5   relative expression of lacI.

1.4   Analysis of transgenic plants for lacI
      expression

      The expression of the lacI gene was monitored
using Western analysis of extracted proteins.
10   Extracts were prepared, proteins recovered on
polyacrylamide gels and prepared for Western
analysis.   The analyses confirmed the expression of
the lacI gene construct in the transformed plants.
Different levels of lacI gene expression were
15   observed in different independent transformants.
The results for plants transformed with the
CaMV-lacI construct are given in the following Table
IV.

TABLE IV	
Plant Sample	Lac Expression (Band Intensity)
L1	−
L2	++
L3	−
L5	−
L7	−
L8	+++
L9	+
L10	+
L11	−
L13	+
L14	++
L15	−
L16	+++
L18	−
L22	+/−
L23	−
L24	++
L25	+
L26	+++
L27	+++
L28	−
L29	+++++
L31	++
L32	++++
L33	+/−
L34	+/−
L35	++
L37	+

− → ++++++ indicates band intensity
of Lac repressor from
Western blots.

2. Insertion of the lac operator into target genes

(a) The maize CAB promoter

The maize CAB promoter can be found in plasmid
pCAB48.1 and we have found that this promoter can
5   drive expression of foreign genes in a transient
tobacco expression system and in stably transformed
plants. This gene, therefore, is an excellent
target to demonstrate control through lacI as high
levels of expression can be obtained both _in vitro_
10   and _in vivo_. Secondly, the CAB promoter from
other systems (wheat, pea and tobacco) have been
extensively analysed in detail and reported in the
literature. The published information facilitates
the selection of suitable sites for operator
15   insertion. Thirdly, pCAB48.1 is a maize promoter
and the use of this system is important to
demonstrate the applicability of this invention to
monocotyledonous plants such as maize,wheat, barley
and sorghum.

20 (b) Insertion of the lac operator
into the maize CAB promoter

Relatively little work has been reported
concerning the characterisation of the important
cis-acting elements of the CAB promoter.
25   Therefore, a computer search comparing consensus
upstream regulatory elements (UREs) of several
plant genes against the CAB promoter was carried
out. As anticipated, numerous putative UREs were
found in both strands of the CAB promoter. A
30   number of potential sites for operator insertion
were selected.

1.   5' of the CAAT box;

2.   Between the CAAT and the TATA box;

3.   Around the TATA box;

4.   Between the TATA box and the transcription
      start point; and,

5.   Between the transcription start point and the
      translation start point.

5   Two methods were used to insert the lac
operator into the maize CAB promoter:
(1) insertion into naturally occurring restriction
sites;
(2) using PCR to introduce operators at selected
10   sites.
          These methods were used to insert lacI
operators into the selected sites.

Method (1)

          Analysis of the promoter sequence shows that
15   this region does not contain many unique
restriction sites.   However, two sites can be made
available by simply recloning the promoter region
into various vectors.

(a) Insertion between TATA and TSP

20   The restriction enzyme PvuII recognises a
single site within the 2.8 kb PstI fragment
containing the CAB gene.   The site lies between
the TATA element and the transcription start point
(TSP) of the CAB promoter.   However, the vector
25   pCAB48.1 contains numerous PvuII sites (within
pUC19).   Therefore, the 2.8 kb PstI fragment was
cloned into the standard cloning vector pAT153
(which lacks a PvuII site) to give pCABP1.

          Operator sequences were inserted into the
30   unique PvuII site within pCABP1.   After
sequencing, it was possible to determine which
clones contain single and tandem operator
insertions.   The synthetic symmetric lac operator
required for this work is shown below and is an 18

base pair palindrome that is analogous to a mutant operator which binds lac repressor eight times more strongly than the wild-type operator.

lac operator-1

5   5' -ATTGTGAGCGCTCACATT- 3'

(b) Insertion upstream of the CAAT sequence

The method used was as follows:

(i) pCAB48.1 was digested with HindIII, which cuts outside the promoter region and within pUC18, and

10   BglII, which cuts downstream of the unique NcoI site and within the coding region.   This gave a fragment with a unique SphI site upstream of the CAAT moiety;

(ii) pUC18 was digested with HindIII and BamHI and

15   the promoter fragment from (i) above was inserted to give pCABP2.   Digestion of pUC18 with BamHI removes the single SphI site from the polylinker. Therefore, pCABP2 contains a unique SphI site into which operators can be inserted.

20   The operator used in this procedure had the sequence:

lac Operator-2

     5'-ATTGTGAGCGCTCACAATCAT G-3'

3'-GTACTAACTCTCGCGAGTGTTA-5'

25   It is important to note that in approaches (a) and (b) the operator sequences are not inserted directly into any putative regulatory elements although promoter activity is likely to be affected when the sequences are inserted elsewhere.

30   Method 2

As shown above, operator sequences can be inserted into two available restriction sites. Insertion into other sites requires other methodologies.

Insertion between TSP and ATG codon

This can be effected using PCR. Since a
unique PvuI site lies close to the TSP region, it
is used as a reference point for subcloning
purposes. The starting material for the PCR
reaction is pCABP1, that is, the pAT 153 CAB
promoter vector constructed as described above. An
oligonucleotide overlapping the PvuII site and
containing no alterations was used to prime the
reaction from one end:

CAB Oligonucleotide-1

    <u>PvuII</u>

5'-GG CAGCTG CTGTGTTCTGTTATGAC-3'

The second oligonucleotide overlaps the NcoI
site and contains the operator sequence shown
below.

CAB Oligonucleotide-2

    <u>NcoI</u>   <u>Operator 1</u>

5'-GATAG CCATGG TGGCGGCAGCCATGTCG ATTGTGAGGCGCTCACAAT

--ATCAGATCGTAGCTCCTTCTGATGC-3'

CAB Oligonucleotide-3

    <u>NcoI</u>   <u>Operator 1</u>

5'-GATAG CCATGG TGGCGGCAGCCATGTCG ATTGTGAGGCGCTCACAAT

    <u>Operator 2</u>

-ATTGTGAGCGCTCACAAT ATCAGATCGTAGCTCCTTCTGATGC-3'

Following the PCR reactions, the newly
synthesised DNA is cleaved with PvuII and NcoI. The
fragment is then transferred to similarly digested
pCABP1 and sequenced.

A slightly different approach which eliminates
the intermediate cloning step into pCABP1 may also be
used. This involves using an oligonucleotide which
overlaps the unique XbaI site in the CAB promoter
together with the operator nucleotides outlined

previously.   Digestion of PCR DNA with XbaI/NcoI
results in a fragment which can be directly cloned
into pCG1 and pCG2.   However the XbaI to NcoI
fragment from the PCR reaction is much larger than the
PvuII to NcoI fragment obtained from the previous
strategy.

Operator insertion between the CAAT and TATA
     This is effected using PCR.
CAB Nucleotide-4
                              XbaI
     5'-CCCAAACAG TCTAGA TATGTTTCTC-3'
CAB Nucleotide-5
                         PvuII   Operator
     5'-CAGAACACAG CAGCTG CCTTTTATAC ATTGTGAGCGCTCACAAT-
-AGTTGGGTTTGGATAGCAGGTCATC-3'
CAB Nucleotide-6
                         PvuII   Operator 1
     5'-CAGAACACAG CAGCTG CCTTTTATAC ATTGTGAGCGCTCACAAT-
          Operator 2
-ATTGTGAGCGCTCACAAT AGTTGGGTTTGGATAGCAGGTCATC-3'
     Following PCR, DNA is digested with XbaI and PvuI
and cloned into similarly digested pCABP1.   Clones
are again characterised by sequencing and any
appropriate DNAs are digested with XbaI and NcoI and
cloned into pCG1 and pCG2.   The basic structure of
these vectors is shown in Figure 11.
The CAMV 35S promoter
     We have found that a promoter-less 35S vector is
an excellent receptor for the insertion of activating
sequences.   The lac operator can be inserted into
this vector, p-Δ-35S, and once inserted the 35S
enhancer is cloned 5' upstream of the lac operator.

(3) Control of gene expression by lac repressor
(a) Control of target gene expression in a transient expression system

Plants which express lacI constitutively transformed with p35lacI may be prepared from protoplasts and, using methods described above) they may be tested for expression of the lacI protein. The target gene constructs may then be introduced into the protoplasts using standard methods and protocols. Protoplasts from untransformed plants can serve as control.   Further control may be provided by protoplasts from plants expressing the GUS marker gene under the control of the CAB promoter without the operator insertions.

(b) Induction of gene expression using IPTG

IPTG can be used to overcome repression by the lac repressor.   Thus, there is formed a switchable gene system.

(c) Modulation of expression of the target gene

Lac repressor/operator interactions can down-regulate marker gene expression in plants to different levels.   This is an important effect in that there may be situations where a different degree of down-regulation may be required.

(d) Control of target gene expression in stably transformed plants

Having shown, as described above, that the lac-repressor can down-regulate CAB promoter driven GUS expression in protoplasts , suitable operator insertion constructs may be transferred to tobacco plants by the methods described above.   The regenerated plants may be crossed with the lacI expressing plants described above, which express the

lac repressor under control of the constitutive
CaMV35S promoter.

Plants may also be constructed which express the
lacI gene under control of the light-inducible maize
CAB promoter.   The expression of the lacI gene in
these plants will then be light-inducible.   These
plants may be crossed with plants which contain the
GUS marker gene from the CaMV promoter containing the
lacI operator insertion.

Insertion of multiple operators into the CAB promoter

Using similar techniques as described for
insertion of single operators, multiple operators can
be inserted into the target promoter. This can either
be by the insertion of multiple copies of the operator
at one site, or the combination of fragments of the
promoter in which the operator is inserted at
different positions in the promoter, this yielding
vectors in which the multiple operators are located at
multiple locations in the promoter.

SECOND EMBODIMENT

In this embodiment the method of regulating gene
expression comprises locating within or inserting into
a gene a pseudo-operator sequence, and providing a
mutant regulatory gene encoding a repressor having an
amino acid sequence which binds to the
pseudo-operator.

Cells containing interacting repressor and
operator genes may be isolated by a method comprising
preparing a recombinant plasmid containing (1) the
Escherichia coli lac operon, which includes the lacZ,
lacY and lacA genes, and (2) a gene encoding a
repressor protein, inserting said plasmid into a
bacterial host and culturing same in the presence of

ortho- or para-nitrophenyl-1-thio- β-galactosidase, whereby the growth of cells in which expression of the lac gene is not repressed by the said repressor molecule is inhibited whereas the growth of cells in which repressor/ operator binding occurs is not so inhibited, and recovering cells displaying non-inhibited growth characteristics.

Mutant repressors may be used or an exogenous potential pseudo-operator may be inserted within the operator region of the lac operon. The exogenous potential pseudo-operator is preferably of plant origin.

A convenient bacterial host is Escherichia coli.

Thus, we have a means for altering the repressor of gene expression enabling genes to be inactivated. Pseudo-operators are DNA sequences which maintain the overall dyad symmetry of an operator but which contain different constituent bases. Computer analysis of known DNA sequences of the French bean GPAL2 gene among many others, and promoter and the mammalian c-myc genes, has revealed a number of possible pseudo-operators to different bacterial repressors.

[The plasmid pGAL2 has been deposited in Escherichia coli strain DH5 on 6th December 1988 with the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, under the Accession Number NCIB 40087.]

Thus, it is probable 'pseudo-operator' sequences can be found in all genes. In general, then, a pseudo- operator is a DNA sequence present at a suitable position in a gene, including a plant gene, at which repressor binding will lead to inhibition of gene expression.

The plasmid pAD18 has been deposited, under the

terms of the Budapest Treaty, in an <u>Escherichia coli</u>,
strain DH5α, host, with The National Collections of
Industrial and Marine Bacteria Limited, Aberdeen,
United Kingdom, on 21 December 1988, under the
5   accession Number 40096.

The plasmid pPS1 has been deposited, under the
terms of the Budapest Treaty, in an <u>Escherichia coli</u>,
strain DH5α, host, with the National Collections of
Industrial and Marine Bacteria Limited, Aberdeen,
10 United Kingdom, on 21 December 1988, under the
accession Number 40097.

This procedure is also applicable to protein
molecules which lead to an increase in gene activity,
particulary the selection of repressors/activator
15 proteins which respond to specific chemicals. Binding
domains for these chemicals can be selected and
specifically manipulated to allow the generation of
specific protein/DNA effect chemical combinations
which are of use in biotechnology, for example as a
20 chemical switch package enabling the controlled
regulation of plant genes by application of an
exogenous chemical inducer.

Mutations which affect both repressors and
operators occur <u>in vivo</u>. It has been shown that
25 repressors which have altered DNA recognition
specificities can be engineered <u>in vitro</u>. The
procedure, then, depends on the ability of rare
repressor mutants to switch off a conditionally lethal
gene by binding at pseudo-operator sequences which the
30 native repressor cannot recognise.

One embodiment of the invention will now be
described, by way of illustration, in the following
example, with reference to the accompanying drawing

which shows a map representing two series of plasmids designated pPS and pAD and variants.

EXAMPLE

We demonstrate the selection system of the invention on repressor phage 434. However, in principle any other repressor can be adapted for this selection system.

1. The selection system

We have designed a selection system that can be used for the selection of mutants in a wide range of repressor-operator systems. The selection system comprises a set of plasmids and the appropriate E. coli hosts, as well as a suicide substrate selection protocol adapted for the plasmids and hosts.

In its final form the system depends on the ability of rare repressor mutants to switch off a conditionally lethal gene through the binding at a 'pseudo-operator' which the wild type repressor cannot bind. The selection system described below contains features which maximise the frequency of such repressor mutants to be identified in the final population of cells.

The selection procedure is based on the lac operon of Escherichia coli and the use of the suicide substrate para-nitrophenyl-1-thio-β-D- galactoside (TPNPG). The lac operon (which contains the three genes lacZ, lacY and lacA) is controlled by the binding of LacI repressor to an operator sequence, lacO, situated between the transcription start site and the lacZ gene. The lacY gene product, lactose permease, is responsible for the active uptake of lactose and related compounds into the cytosol where they are hydrolysed by β-galactosidase (the lacZ gene product) to form galactose and glucose.

The positive selection system exploits the
discovery that the growth of cells expressing the lacY
gene is selectively inhibited in the presence of TONPG
or TPNPG, presumably through the waste of metabolic
5   energy on its transport. The selectivity of these
compounds has been shown to be enhanced when succinate
is used as the carbon source.

The rationale behind the selection is based on
the ability of 434 repressor to bind the
10   pseudo-operator sequences inserted in the promoter
driving expression of the lac gene cassette. In the
absence of a 434 repressor/operator complex, the lac
operon will be expressed and, in the presence of
TPNPG, will result in cell death. Conversely, in the
15   presence of a complex, the LacY permease will not be
expressed, and the suicide substrate TPNPG will be
unable to enter the cell. Consequently, in the final
analysis, a pseudo- operator chosen from the natural
sequence of the target plant gene will be cloned into
20   the SalI site and combined with a pool of genes
encoding 434 repressors in which certain amino acids
in the α3 helix are randomly substituted. Only those
cells expressing mutant repressor that is able to bind
the pseudo-operator, and consequently repress lacY
25   expression, will be selected in the presence of TPNPG.

2. The plasmids

2.1 Construction of pAD18 and Derivatives

A series of plasmids have been developed for use
in these experiments. The prototype of these is
30   pAD18, a map of which is shown in Figure 13. This
vector is based on a replicon from pSC101, which is
known to be stably maintained in E. coli, and to have
a low copy number. This is important as
overexpression of DNA binding proteins may have

deleterious effects on the growth of the host. If this is a problem in some experiments, it is advisable to transfer genes contained on the pAD18 to a bacteriophage vector for insertion into the bacterial genome as a single copy gene.

pAD18 has a kanamycin selectable marker for maintainance in E. coli strains.

pAD18 also contains the lac operon. The lacZ and lacY genes are present under the control of the lac promoter/operator. Into the lac operator, a SalI restriction site has been enginered which is used for insertion of the 434 operator, or in derivatives of pAD18, mutant 434 operators or selected 'pseudo-operators'. This site has been positioned in such a way that it will not interfere with expression of the lac operon from the lac promoter through steric hinderances when sufficient repressor is synthesised to bind to the operator cloned into that site. Those bases were changed into SalI restriction sites which are known not to be involved in contact with RNA polymerase. Thus the lac operon expression will be manipulated under the control of the 434 repressor. pAD18 which contains the wild-type 434 operator is thus the prototype of this series of plasmids.

pAD18 contains a tetracycline resistance gene, into which a wild type 434 repressor gene under the control of the lacUV5 promoter can be inserted for high level of expression. This vector is called pPS1. Further derivatives are described below.

In another vector, the 434 repressor has been modified such that a Kpnl and Notl site has been introduced at either side of the DNA binding helix whilst the native amino acid sequence in this region has been preserved. It is thus be possible to insert

into this 434 repressor gene random oligonulceotides
which when expressed will generate 434 repr ssor
molecules which express altered DNA binding domains.
The selection system using a suicide substrate will
then permit the selection of those 434 mutant
repressors which bind to the 'pseudo-operator'. In
some circumstances this may also provide the selection
pressure for isolation of repressor mutants. However,
the system as it stands is dependent on
expression/repression of the lacY permease for the
isolation of repressor mutants.

    Thus, there are convenient cloning sites in pAD18
and its derivatives for insertion of operators or
repressor genes. Operators can be cloned into
precursor vectors of pAD18, especially pRW283, from
which the operator containing EcoR1 to Pst1 fragment
can subsequently be excised and cloned into EcoR1 and
Pst1 digested pAD18 (see Figure 13).

    One objective was to show that the expression of
the lac operon carried by the plasmids described above
could be controlled by 434 repressor/operator
interactions. To demonstrate this, three plasmids
were constructed in which the 434 operators carried by
pAD16, 17 and 18 were combined with the wild-type
434cI gene on pAD15.2. The large XhoI/SstI fragment
(9.4kb) from pAD15.2 was purified and ligated to the
small XhoI/SstI fragment (3.7kb) purified from pAD16,
17 and 18 to form plasmids pPS2, pPS3 and pPS1,
respectively. Restriction analysis of plasmid DNA
isolated from several transformants from each ligation
showed that all pPS plasmids had the expected overall
structure. The structure of these plasmids is shown
in Figure 13.

    The integrity of the operators carried by the pAD

and pPS plasmids was checked by sequencing. Initially
this was achieved by isolating the approximately 200
base-pair EcoRI/PstI fragment, which carries the whole
of the lac promoter and the 5' end of the lacZ gene,
from each of the pAD and pPS plasmids and subcloning
them into the polylinker of M13mp18. Single-stranded
template was purified and sequenced according to
standard protocols. Alternatively, this laborious
subcloning procedure was circumvented by using plasmid
sequencing. These analyses showed that the
appropriate 14mer operator sequence was present at the
SalI site in all relevant plasmids. The presence of
other salient features of the lac promoter was also
confirmed.

2.2 <u>pPS plasmids encode functional 434 repressor.</u>
To visualise the 434 repressor produced by
pAD15.2 and the pPS plasmids, total protein extracts
were prepared from mid-log cultures grown under
selective conditions. Following polyacrylamide gel
electrophoresis and Coomassie brilliant blue (G250)
staining, no proteins corresponding to the size of the
repressor could be observed specifically in strains
containing the 434cI gene. However, other experiments
have shown that 1μg of purified repressor is only just
visible using this relatively insensitive technique.
Therefore, to detect 434 repressor in the amount of
extract used an expression level of at least 1% total
cell protein would be required. The background of
other similar-sized proteins also makes detection
difficult. Consequently, the much more sensitive
Western blotting technique was used. The primary
antibody required to detect 434 repressor by Western
blotting was prepared by injecting rabbits with

purified intact 434 repressor. The specificity of
this polyclonal antiserum was demonstrated using
purified repressor and extracts of E.coli strains
harbouring the 434cI gene. At low dilutions of
5   antiserum several proteins from bacterial extracts,
including 434 repressor, were detected. However,
further dilution of the antiserum resulted in only 434
repressor remaining detectable, the maximum
specificity being observed at dilutions of 1/10000 to
10   1/20000.

The sensitivity of Western blotting using this
antibody preparation and the horse-radish peroxidase
conjugate detection technique was assessed by
"spiking" crude cell extracts of 6300Δlac4169, which
15   contains no 434cI gene, with various amounts of the
purified repressor. Under standard conditions, 5ng of
repressor in 50µg of extract could readily be
detected, this sensitivity corresponding to an
expression level of 0.01% total cell protein.

20   Use of the same primary antibody in Western
blots of 6300Δlac4169 strains carrying the test
plasmids showed conclusively that cells harbouring
pAD15.2; pPS1, pPS2 and pPS3 all synthesised 434
repressor. Determination of the relative intensities
25   of the bands obtained, using a scanning densitometer,
showed that all four strains contain approximately
0.4% total cell protein as 434 repressor.

Finally, the ability of the 434 repressor to bind
wild-type operator sequences was determined in a
30   functional assay using bacteriophage 434cI, 434vir and
λcI. In the life cycle of these phage, the binding of
the appropriate repressor to operators within the
promoter P_R represses transcription of the genes

responsible for cell lysis. Consequently, cells which
endogenously synthesise cI repressor are immune to
lysis by the corresponding phage due to the inhibition
of lytic gene expression by the already existing

5   repressor. Since the cI mutant phage are unable to
synthesise repressor, the lytic phenotype after
infection by these phage is diagnostic for the absence
of repressor in the host cell. The P_R operators of
vir mutant phage have a reduced affinity for

10   repressor, with the consequence that at low levels of
endogenous repressor this phage is lytic, whereas at
higher repressor concentrations super-infection is
inhibited.

The results of cross-streaking these phage with

15   the test strains show that cells harbouring the pPS
plasmids are immune to super-infection by both 434cI
and 434vir, but are sensitive to λcI. Strains
carrying other pAD plasmids were sensitive to all
three phage. This clearly indicates that cells

20   carrying the pPS plasmids synthesise high levels of
434 repressor that is functionally able to bind
operator and inhibit transcription from P_R.
Furthermore, the specificity of this
repressor/operator interaction is demonstrated by the

25   inability of 434 repressor to bind the operators of
λcI, which have a different sequence to those of 434,
resulting in cell lysis.

In summary, all three pPS plasmids were
determined to be of the correct construction, to carry

30   the expected 434 operator sequence and to synthesise
functional 434 repressor.

2.3 VECTOR IMPROVEMENT

As indicated below, a proportion of the
population of pAD and pPS plasmid-containing strains

form white colonies upon selection. Furthermore, it
has been observed that if strains containing these
plasmids are kept on selective media for several
months, sub-culturing when necessary, the proportion

5   of white colonies in the population increases.

It is presumed that these white colonies carry
plasmids in which part of all of the lac operon is
mutated or deleted. The usual method of minimising
such problems is to use a recombination-deficient

10   strain. However, the combination of the Δlac4169 and
recA56 alleles renders the strain inviable in the
presence of TPNPG, the reason for this being unclear.
Therefore, attention has turned to the probable source
of the recombinatory events. It is noted that the

15   promoters expressing the lac operon and the 434
repressor gene in the pPS plasmids are both derived
from the lac promoter and consequently the sequences
are very similar. Recombination between these
sequences would result in the deletion of the lac

20   operon, given that the origin sequences and the
kanamycin-resistance gene must remain under the
selective conditions imposed.

During the construction of pAD15.2, the 434cI
gene was transferred from plasmid pRP42 on a 1kb Sau3A

25   fragment. The stop codon of the 434 repressor reading
frame coincides with the Sau3A site at the right hand
side of this fragment, consequently the gene cloned
into pAD15.2 carries no transcriptional termination
signals. Furthermore, this Sau3A fragment also carries

30   a remnant of pBR322, including the ampicillin-
resistance gene promoter. Therefore, to rectify these
problems sequences both upstream and downstream of the
434cI coding sequence were altered. The region
upstream was replaced with the tryptophan promoter and

a consensus Shine-Dalgarno sequence using appropriate
oligonucleotides. This both prevents intra-plasmidic
recombination and removes the ampicillin-resistance
gene promoter.

The rrn T1 terminator was also introduced at the
3' end of the 434cI coding sequence to terminate the
434cI gene transcripts. This rho-independent
terminator was chosen since its reported
bi-directional termination activity would avoid any
disruption of 434cI expression from opposing
transcripts initiated elsewhere in the vector as well
as terminating 434cI transcripts.

2.4   Construction of plasmid pTP1.

Oligonucleotides were used to introduce the
wild-type trp promoter sequence together with the
consensus Shine-Dalgarno sequence (AGGAGGT) 5
base-pairs upstream of the 434cI gene translational
start site. This spacing gives maximal translational
activity of the 434cI gene. Due to the lack of
convenient restriction sites at the start of the 434cI
gene, the EcoRI site 33 base-pairs inside the coding
sequence was used. This necessitated the inclusion of
the 5' end of the coding region in the
oligonucleotide. The required sequence is 126
base-pairs long and thus was constructed from four
overlapping oligonucleotides. Following the
annealling of these oligonucleotides, the duplex 126
base-pair fragment was isolated and cloned into
EcoRI-cleaved pUC19 vector. Following the selection
of transformants on media containing ampicillin and
BCIG, DNA from several white colonies was sequenced
using plasmid sequencing protocols. This confirmed
the structure of the promoter sequence to be as
expected.

2.5   <u>Construction of pTT1.</u>

      The sequence of the rrn T1 terminator has a long
G ≡ C rich stem structure flanked by long A=T rich
regions making it a strong terminator for transcripts
5   in both orientations. Due to the inverted repeat
nature of this sequence, it was inserted using four
oligonucleotides thereby avoiding any problems of
self-annealling within each strand. The
oligonucleotides were annealled pairwise and the
10   resultant double-stranded DNAs isolated separately and
ligated to EcoRI/HindIII cleaved pTP1 DNA, prior to
transformation of cells to ampicillin-resistance. The
sequence of the inserted terminator structure was
ascertained.

15   2.6   <u>Construction of plasmid pTRT1</u>

      The source of the 434cI gene to be cloned behind
the trp promoter was plasmid pRP42-76. Silent
mutations have been introduced, using in vitro
mutagenesis, to create restriction sites for KpnI and
20   XmaIII on either side of the sequence coding for the
α3 recognition helix. This will subsequently allow
the introduction of oligonucleotides in which certain
codons in the α3 helix have been randomly mutated.
The EcoRI/Sau3A fragment (approx. 600 base-pairs)
25   carrying the 434cI gene was isolated and cloned into
EcoRI/BglII cleaved pTP1. This reforms the 434cI open
reading frame exactly and the translational stop codon
is retained within the Sau3A/BglII junction. Once
isolated, the trpP-434cI-rrnT1 cassette was cleaved
30   out using the BamHI sites in the polylinkers
introduced at either end of the cassette and used to
replace the existing 434cI gene in the pAD and pPS
plasmids.

The sequence of the trp promoter used to construct pTRT1 is bound by trp repressor in the presence tryptophan to inhibit transcription. The binding site for this repressor was intentionally retained in order that expression of the 434cI gene may be controlled, if necessary, in future work. However, in order to conveniently allow the synthesis of 434 repressor, strains will be constructed in which the trpR gene has been deleted from the chromosome.

3. Selection Protocol

3.1 Selection using TONPG

A TONPG (ortho-nitrophenyl-b-thiogalactoside) selection protocol was designed which allows selection for clones in which a mutant repressor now binds to the mutant 434 operator resulting in repression of lacY expression (i.e. selection by repression of conditional inhibition).

TONPG inhibits the growth of E. coli cells that are expressing the lacY permease gene. Early work with single copy lacY+ E. coli indicated that these cells were maximally sensitive to TONPG at 500 to 1000 micrograms/ml when expressing lacY in succinate minimal medium. Mixing experiments showed that TONPG could be used to select lacY cells from a mixed lacY+/- population. These experiments were repeated with lacY+ and lacY- pAD plasmids. The TONPG selection will only work in a lac-deleted E. coli host. The preferred host is described below. Selection works better in liquid cultures, but also works on agar plates. Selection on solid medium works better with a different galactoside analogue, para-nitrophenyl-beta- thiogalactoside (TPNPG). Using TONPG in liquid cultures and TPNPG in plates, the

selection normally achieves a 6 log enrichment of
lac-pAD plasmids present in the initial population.

3.2   The E.coli host

5   The bacterial host selected was such as to enable
selection by TONPG. This required the ability of the
host to grow in a succinate minimal medium. The
particular host used in this Example was one from
which the entire lac operon had been deleted,
lacΔ4129. However, other suitable mutants hosts can
10   be used, for example laci$^-$, lacY$^-$. A suitable strain
was constructed from strain W1485 (CGSC6300)
derivative which was deleted for the entire lac
operon, using transposon-linked P1 transduction.

3.3 Selection using TPNPG

15   To test the ability of TPNPG to select lac$^-$
cells from a background of lac$^+$ cells, mixing
experiments with cultures of 6300lac$^+$ and its Δlac4169
derivative were performed. These strains were grown
to mid-log phase in the presence of 1mM IPTG, to
20   induce expression of the lactose operon. The two
cultures were mixed in various proportions before
plating suitable dilutions on minimal-salts-succinate
plates containing 1mM IPTG and 50μg/l BCIG, both with
and without TPNPG.

25   Initial experiments showed that 500μg/ml TPNPG
was only able to retard the growth of lac$^+$ cells,
allowing the formation of small blue colonies after
48h at 28°C. However, this background was eliminated
in the presence of 1mg/ml TPNPG, resulting in none of
30   the lac$^+$ cells plated (up to 7×10^{-7}) being able to
form blue colonies (Table V). In contrast, this
TPNPG concentration did not noticeably affect the
viability of the lac$^-$ cells. This demonstrates the

high selective power of TPNPG against lac$^+$ cells, even when the lacY gene is chromosomal and thus at a low copy number. In this respect, the higher copy number and therefore increased expression level of the lac genes on the pPS plasmids should result in a greater waste of energy on TPNPG uptake, making the killing of lac$^+$ cells more effective.

TABLE V

ABILITY OF TPNPG TO SELECT LAC- CELLS FROM A BACKGROUND OF LAC+ CELLS				
Approx. ratio of cells plated lac-:lac+	Colonies formed/ml			
	-TONPG		+TONPG	
	White	Blue	White	Blue
1:10^2	1.1x10^2	2.0x10^4	1.2x10^2	0
1:10^3	1.3x10^2	2.0x10^5	1.2x10^2	0
1:10^4	N.D.	1.8x10^6	1.1x10^2	0
1:10^5	N.D.	1.5x10^7	1.1x10^2	0
1:5x10^5	N.D.	7.0x10^7	1.2x10^2	0

N.D. = Not Determinable

The effect of TPNPG on the survival of
6300Δlac4169 cells carrying various pAD and pPS
plasmids was tested by plating appropriate
dilutions of cultures on the media as described
5   above. The results from two experiments revealed
that all plasmids resulted in the formation of both
blue and white colonies in the presence of TPNPG,
yet no white colonies were detected in its absence
(Table VI).

TABLE VI

Plasmid carried	Colonies/ml			
	−TPNPG		+TPNPG	
	White	Blue	White	Blue
Expt#1				
−	2.8×10^8	0	3.0×10^8	0
pAD16	ND	3.5×10^8	1.2×10^3	2.0×10^3
pAD17	ND	2.3×10^8	1.3×10^4	1.6×10^3
pAD18	ND	2.3×10^8	2.3×10^3	2.2×10^3
pPS1	ND	3.8×10^7	2.0×10^4	2.7×10^7
pPS2	ND	7.2×10^7	7.0×10^2	4.8×10^7
pPS3	ND	5.6×10^6	2.0×10^4	2.0×10^1
Expt#2				
−	3.1×10^8	0	3.0×10^8	0
pAD16	ND	2.9×10^8	1.1×10^4	4.2×10^2
pAD17	ND	3.4×10^8	6.7×10^3	5.9×10^3
pAD18	ND	2.7×10^8	4.1×10^3	2.5×10^3
pPS1	ND	4.2×10^7	8.5×10^3	1.2×10^7
pPS2	ND	8.4×10^7	5.2×10^3	1.6×10^7
pPS3	ND	5.8×10^7	5.7×10^3	6.3×10^1

ND = Not Determinable

As already observed, all pAD and pPS plasmids give blue colonies on media containing BCIG, irrespective of the presence of 434 repressor/operator interactions. Presumably therefore, the white colonies formed must result

from cells carrying plasmids which have been
mutated or deleted to render the cell effectively
lac⁻. The relatively low frequency with which
these white colonies occur (approx. 10^{-5} of cells
5   plated) suggests that, on media lacking TPNPG, they
would remain undetected amongst the majority of
blue colonies. Analysis of the plasmids harboured
by cells of such white colonies revealed deletions
(see above).

10   In the presence of TPNPG the frequency of
blue colonies formed by pAD-carrying strains was
reduced by 10^5 to 10^6. This represents the killing
by TPNPG of the majority of the population
harbouring an un-repressed lac operon. The strain
15   carrying pPS3 was also killed to a similar extent
in the presence of TPNPG, as would be expected
given that it has already been shown that the 434
repressor is incapable of inhibiting transcription
of the lac genes in this plasmid. However, in all
20   cases, a residual number of blue colonies were
obtained in the presence of TPNPG, at a frequency
of approximately 10^{-5} of the cells plated. It is
presumed that these colonies primarily represent
cells harbouring lacY⁻ mutant plasmids, since it
25   has already been demonstrated that the selective
power of TPNPG is sufficient to kill the vast
majority of the cells plated, given that they all
remain lac⁺. Since previous experiments have not
indicated a high mutation frequency for the
30   chromosomally borne lacY gene, it is presumed that
intra-plasmidic recombination is responsible for
the apparently high number of mutants. Previous
work has indicated that these plasmids are prone to

instability in rec$^+$ strains and that a 6300Δlac4169
recA56 strain, which could be used to prevent such
recombination, is inviable on TPNPG.

However, in sharp contrast to the other
strains, the vast majority of cells containing pPS1
or pPS2 survive in the presence of TPNPG, the
number of blue colonies being reduced by only 2-5
fold, this reduction being least for pPS1. This
clearly correlates with the emphatic reduction in
β-galactosidase activity, and therefore also
presumably lacY expression, already demonstrated
for these plasmids. Therefore, the important
conclusion can be drawn that the interaction
between 434 repressor and its cognate operator is
able to reduce lac gene expression sufficiently to
allow the majority of cells to survive the
selective procedure.

4. Selection of altered specificity repressors
4(a) Selection of altered 434 repressor

A 434 gene which had been altered to
facilitate random mutagenesis of the 434 repressor
binding domain through insertion of random
oligonucleotides has been described (Wharton and
Ptashane, Nature 316, 601-605). The 434 repressor
gene has been mutagenized to introduce KpnI and
NotI restriction enzyme cleavage sites on either
side of the DNA recognition helix, whilst
conserving the native amino acid sequence. Batches
of oligonucleotides have been synthesised with the
correct cohesive ends and containing varying
frequencies of mutations randomly distributed
throughout the DNA recognition alpha helix. These
oligo mixtures have been cloned between the KpnI
and the NotI cohesive ends of the modified 434

repressor gene. Alternatively the 'dirty oligo'
approach has been used for the generation of mixed
oligo with base substitutions at appropriate
positions of the DNA binding domain.

5   4(b) Selection of altered 434 repressor
recognising pseudo-operator found in plant genes
A naturally occurring pseudo-operator was used for
the selection of altered repressor. The target for
this work was the GPAL2 gene from French bean, the
10   chlorophyll a/b binding protein gene from maize,
and others. We have identified by computer
analysis, that several potential 434
pseudo-operators are located in the region of the
GPAL2 gene. These regions of the GPAL2 promoter
15   were used to select an altered specificity 434
repressor. The 'pseudo-opertor' sequences were
inserted into the -10 to -35 region of the lac
promoter driving the lacZ/lacY genes. Dirty
oligo's were inserted into the 434 repressor gene
20   and mixtures were transformed into E.coli 6300.
The selection protocol was applied and colonies
isolated. Using microbiological and molelcular
techniques we have demonstrated that mutant
repressors can be selected for. The
25   characterisation of the repressor gene has been
done by DNA sequence analysis, and binding studies
to determine the strength of the repressor binding.
In summary, then, the present invention
provides a selection system comprising preferably
30   of bacterial strains and plasmids, and a sensitive
suicide substrate selection protocol. This
selection system can be used to select altered
specificty repressors. Implied in this invention
is the provision of controlling gene expression in

organisms by said altered- specificity repressors. The only requirement for this method of control of gene expression are the DNA sequence of the target gene, the identification of 'pseudo-operators' being a DNA sequence that resembles the normal operator sequence and a selection system which permits the selection of repressors capable of binding to said 'pseudo-operators'.

III. MALE FLOWER SPECIFIC GENE SEQUENCES

This module contains male flower specific DNA sequences comprising the polynucleotides shown in Figures 17, 18, and 19 herewith, which are specifically expressed in male flower tissue.

Plasmid pMS10 in an _Escherichia coli_ strain R1 host, containing the gene sequence shown in Figure 17 herewith, has been deposited with the National Collection of Industrial & Marine Bacteria on 9th January 1989 under the Accession Number NCIB 40098.

Plasmid pMS14 in an _Escherichia coli_ strain DH5α host, containing the gene control sequence shown in Figure 18 herewith, has been deposited with the National Collection of Industrial & Marine Bacteria on 9th January 1989 under the Accession Number NCIB 40099.

Plasmid pMS18 in an _Escherichia coli_ strain R1 host, containing the gene control sequence shown in Figure 19 herewith, has been deposited with the National Collection of Industrial & Marine Bacteria on 9th January 1989 under the Accession Number NCIB 40100.

The isolation and characterisation of these cDNA sequences and the utilisation of these cDNA sequences as molecular probes to identify and isolate the corresponding genomic sequences will

now be described.

The clones carrying the genomic sequences and
the preparation of a promoter cassette from one of
the clones illustrated using an approach and
5   techniques which may be equally applied to any of
the the clones. Furthermore the preparation of a
promoter fusion to a reporter gene and the
transformation of this construct into a test
species is described.

10   Unless stated otherwise, all nucleic acid
manipulations are done by standard procedures
described in Sambrook, Fritsch and Maniatis,
"Molecular Cloning: A Laboratory Manual", Second
Edition 1989.

15   EXAMPLE 1

1.   Isolation and Characterisation of Male Flower
      Specific cDNA from Maize

          To clone cDNAs to genes which are expressed
in the male flowers of maize we constructed two
20   cDNA libraries. In maize, the male flowers are
born in the tassel which terminates the main stem.
Library 1 was prepared from poly [A] RNA from whole
maize tassels bearing early meiotic anthers (most
meiocytes in early meiotic prophase) and library 2
25   from poly [A]+ RNA from whole tassels bearing late
meiotic anthers (predominantly diad and early
tetrad stages). Figure 14 reviews the library
screening procedure used and this yielded five
unique early meiotic MFS cDNAs and one unique late
30   meiotic cDNA. Clone PMS3, a partial cDNA of 120
base pairs, isolated by the differential screening
process, was subsequently used as a hybridisation
probe to isolate the corresponding pending near
full-length clone, PMS18.

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Table VII below summarises some of the
features of each of these cDNA clones. Expression
of the mRNAs of the five MFS cDNAs isolated from
the early meiotic library is detected in RNA
5   isolated from both early and late meiotic tassel
samples.   The mRNAs corresponding to these cDNAs
are not wholly specific to male flowers and are
detected at considerably lower levels in leaves
(pMS10 and pMS18) or in leaves, cobs and roots
10   (pMS1, pMS2 and pMS4) Table VII.   In contrast pMS14
mRNA is found only in late meiotic RNA and is not
detected in leaves, cobs or roots (Table VII).

TABLE VII						
	pMS1	pMS2	pMS4	pMS10	pMS14	pMS18
Library1	1	1	1	1	2	1
Insert size2	750	500	720	1350	620	940
mRNA size3	900	950	850	1600	900	1100
Organ specificity4	+	+	+	++	+++	++
Expression window5	E/L	E/L	E/L	E/L	L	E/L

Table Legend

1   Isolated from cDNA library 1 (early meiotic)
       or library 2 (late meiotic.

2   Approximate size in base pairs.

5   3   Approximate size in nucleotides.

4   + = expresed in tassels and at much lower
       levels in leaves, cobs and roots.

       ++ = expressed in tassels only and at much
       lower levels in leaves.

10   +++ = expressed in tassels only.

       5   E/L = mRNA present in RNA from both early and
       late meiotic tassels.

       L = mRNA present only in RNA from late meiotic
       tassels.

15   We have examined expression of the genes
corresponding to these cDNAs during tassel
development using dot blot hybridisations (Figure
15). The dot blot analysis was generated by
binding total; RNA to nitrocellulose followed by
20   hybridisation to radiolabelled pMS cDNAs. All
filters were exposed to film for 48 hours at $-70°C$
except pMS10 which was exposed for 168 hours. The
tassel lengths in each sample were as follows: A $\geq$
2cm; B=2-5cm; C=5-10cm; D=10-15cm; E= 15-20cm;
25   F=20-30cm; and G=20-30cm. The solid bars in Figure
15 show the developmental stage relative to
microsporogenesis in each of the samples: PM =
premeiosis; M = meiosis; IP = immature pollen; and
MP = mature pollen.

30   The early meiotic mRNAs (pMS1, 2, 4, 10 and
18) accumulate very early in development in tassels
less then 2 cm in length. We have not analysed
expression in floral meristems prior to this stage.
These mRNAs persist through the meiotic anther

stages and then decline as pollen grains mature.
In contrast the late meiotic mRNA of pMS14 is not
detected in tassels less then 5 cm in length, but
increases dramatically as the sporogenous cells of
5   the anther enter meiosis (Figure 15). As with the
early meiotic mRNAs, pMS14 mRNA declines abruptly
as mature pollen accumulates in the anthers (Figure
15).

        These data show that different temporal
10   controls of gene expression occur during
development of male flowers in maize. The controls
which programme accumulation of the early meiotic
mRNAs are probably very similar but contrast
markedly with those regulating appearance and
15   accumulation of the late meiotic mRNA, pMS14. Both
the early and late meiotic mRNAs are involved with
developmental processes which occur prior to the
accumulation of mature pollen grains. They are
clearly not involved with the later stages of
20   anther development such as dehiscence nor are they
mRNAs which accumulate in mature pollen.

        The technique of in situ hybridisation has
been used to determine the tissue localisation of
MFs mRNAs in male flowers of maize. The techniques
25   used are described in Wright and Greenland (1990;
SEB Seminar Series, vol 43 ed by N Harris and D
Wilkman. Cambridge University Press, Cambridge; in
the Press). The data shown is that for pMS14 mRNA.

        Figure 16 A,B shows in situ hybridisation with
30   pMS14 antisense RNA probes. Sense and antisense
probes more prepared by sub cloning a 300 basic
pair fragment of pMS14 into the vector, pBS,
followed by preparation of radiolabelled T3 and T7
polymerise transcripts utilising methods suggested

by the supplier of the vector (Stratagene, Trade
Mark). These hybridisations show that pMS14 mRNA
is located in the tapetal cell layer surrounding
the developing microspores. Hybridisation of the
5   pMS14 antisense probe does not occur to any other
cells in the section. Likewise the pMS14 sense
probe does not show any specific hybridisation
(Figure 16C). These sections were made from 15-20
cm maize tassels at a stage when the level of pMS14
10   mRNA is at a maximum (Figure 15). In these
sections and in those from subsequent experiments
hybridisation occurs to the tatetum of the anthers
in one floret but not the other. In Figure 16 A,B
the tapetal layers which contain pMS14 mRNA
15   surround late meiotic microspores at the tetrad
stage whilst the tapetal layers not containing
pMS14 mRNA surround sporogenous cells which have
not undergone meiosis. It is a feature of maize
that the sets of anthers within the individual
20   florets of the spikelet do not develop
co-ordinately. Thus in situ hybridisation shows
that accumulation of pMS14 mRNA is tissue-specific
and confirm data obtained from dot blot analysis
(Figure 15) that expression of PmS14 mRNA is stage
25   specific as it is first detected in tapetum
surrounding meiotic cells.

EXAMPLE 2

Determination of DNA sequence of pMS10

DNA from cDNA clone, pMS10, for sequence
30   analysis by subcloning into M13mp18 using standard
procedures. The nucleotide sequences of the
subclones were determined by the dideoxy method
using standard procedures. In addition a Sequence
(Trade Mark) method was used utilising methods

described by the suppliers. Regions of the clones were sequenced by priming with synthetic oligonucleotides synthesised from sequence obtained from previous gel readings. Oligonucleotide

5   concentrations used for priming were identical to those used with universal primers.

MFS, Clone pMS10 full length cDNA of 1353 base pairs. The complete nucleotide sequence and the predicted amino acid sequence are shown in Figure

10   17. The sequence contains an open reading frame of 1022 nucleotides encoding a polypeptide of 341 amino acids with a deduced molecular weight of 37371 kd the polypeptide is rich in glycine residues. The open reading frame is flanked by 5'

15   and 3' non-translated regions of 129 and 201 bases respectively.

EXAMPLE 3

Determination of DNA sequence of pMS14

Procedure of determining nucleotide sequence

20   as described in Example 2.

Clone pMS14 is an in complete cDNA of 581 base pairs the complete nucleotide sequence and deduced amino acid sequence are shown in Figure 18. The sequence contains an open reading frame which

25   extends from nucleotide 1 to 278 encoding a partial polypeptide of 127 amino acids. The polypeptide is particularly rich in alanine and arginine residues. The open reading frame is flanked by 3' non-coding region 203 nucleotides. A consensus processing and

30   polyadenylation signal hexanucleotide, AATAAA occurs at position 548.

EXAMPLE 4

Determination of DNA sequence of pMS18

Procedure for determining nucleotide sequence

as described in Example 2.

    Clone pMS18 is a near full-length cDNA of 933
bases. The complete nucleotide sequence and
deduced amino acid sequence is shown in Figure 19.
pMS18 lacks 28 nucleotides at its 3' terminus. The
missing nucleotides are present in clone pMJ3 which
overlaps the sequence of pMS18 by a further 91
nucleotides. pMS3 was the original clone isolated
by differential screening of cDNA inbranes and was
subsequently used as a hybridisation probe to
isolate pMS18. pMS18 contains an open reading
frame extending from nucleotide 151 to 813 and
encodes a polypeptide of 221 amino acids with a
deduced molecular weight of 25 kilodartons. The
polypeptide is particularly rich in arginime
residues. The open reading is flanked by 5' and 3'
non-coding regions of 150 and 120 nucleotides
respectively.

EXAMPLE 5

Isolation of genomic clones corresponding to pMS10

    Genomic DNA clones carrying genes
corresponding to the cDNA, pMS10 were isolated from
an EMBL 3 phase library of partial Mb01 fragments
of maize DNA. The library was screened using
radiolabelled "long-mer" probes synthesised in an
in vitro labelling system. This system comprised,
50 mg of a synthetic 100 base oligonucleiotide
(base position 452-551 at pMS10; Figure 17). 500
mg of a synthetic primer olignucleotide, sequence -
TAGTTTCCT-CGGTAG and which will base pair with the
3' end of the long olionucleotide, one or two
radiolabelled oligonucleotides (usually ^{32}PdCTP
and/or ^{32}P-dGTP) and 5-10 units of the Klenow

fragment of DNA polymerase 1. The reactions were
performed at 37°C for 30 minutes in a buffer
identical to that used for the "random-priming"
method of DNA labelling except that the random
hexanucleotides were omitted. Five million phase
clones immobilised on nylon "Hybaid" (Trade Mark)
filters were hybridised at 65°C with these probes
using prehybridisation and hybridisation buffers
suggested by the suppliers of the filters (Amersham
International). Filters were washed on 3 x SSC,
0.1 % SDS at 65°C using these procedures 50-60
EMBL3 phage clones containing either complete or
partial regions of a pMS10 gene were obtained. The
DNA from three EMBL3 phage clones 10/CT8-1,
10/CT8-3 and 10/CT25-3 which combined complete
pMS10 genes was prepared and analysed by
restriction enzyme digests. Each of these clones
was shown to contain a common 9Kb EcoRI fragment
which extends from the third intron of the pMS10
gene into the 5' non-coding and promoter regions of
the gene. A partial restriction map of the 9 Kb
EcoRI fragment is shown in Figure 20.

EXAMPLE 6

Isolation of geomic clones corresponding to pMS14

To isolate genomic DNA clones carrying genes
corresponding to the cDNA, pMS14 two approaches
were taken. In the first approach the method shown
in Example 5 was adopted except the 5 million phage
clones were screened with the complete cDNA
sequence and the wash stringencies after
hybridisation procedure yielded two positive clones
14/CTA and 14/CTD. In the second approach a 12 Kb
EcoRI cut fraction of maize geomic DNA, shown by
Southern Blotting to carry the pMS14 gene, was

ligated into EcoRI cut λ phage EMBL4 DNA to produce
a library of cloned 17 Kb DNA fragments. Roughly
200,000 clones were screened as described above,
and two positive clones, 14/17m and 14/17R which
5   combined a 17 Kb EcoRI fragment which hybridized to
pMS14, were isolated. On further analysis the two
positive clones isolated from the partial
MboI/EMBL3 library were found to contain an
internal 17 Kb fragment. A partial restriction map
10   of this 17 Kb EcoRI fragment, common to all the
clones, is shown in Figure 21.
EXAMPLE 7
Isolation of genomic clones corresponding to pMS18
       To isolate genomic DNA clones carrying genes
15   corresponding to the cDNA pMS18, the procedure
described in Example 5 was adopted. Five million
EmBL3 phage clones were hybridized to a "long-mer"
probe derived from the sequence of pMS18, position
133-222). The sequence of the 3' complementary
20   oligonucleotide was a 5'-GCCTCGGCGGTCGAC-3'. Two
clones, 18/CT3 and 18/CT23, carrying the pMS18 gene
were isolated from this screen. Restriction
mapping of these clones showed that they both
contained a 4.5 Kb BamHI-SalI fragment comprising
25   the 5' region of the coding sequence of pMS18 and
approximately 4 Kb of the promoter and upstream
region of the gene. A partial restriction map of
clone 18/CT3 is shown in Figure 22.
EXAMPLE 8
30   Construction of a promoter cassette derived from
10/CT8-3
       The following subclones from the λEMBL3 clone
10/CT8-3 were made. The 4.5 Kb PstI-EcoRI fragment
was cloned into pUC18 to give pMS10-2. The 2.7 Kb

XbaI-EcoRI fragment was cloned into pUC 18 to give
pMS10-3. The 1.6 Kb HindIII to XbaI fragment was
cloned into pUC18 to give pmS10-4.

    The polymerase chain reaction (PCR) was used
to amplify a 930 bp fragment from pMS10-3. The
primers used for the PCR reaction were as follows.
Primer pUC/2 is homologous to pUC sequence flanking
the polylinker site. Primer 10/9 is complementary
to the sequence of pMS10 from position 106-129
except that it contains an additional thymidine
residue between bases 123 and 124. The sequence of
these primers is:

      pUC/2 5' CGACGTTGTAAAACGACGGCCAGT-3'
      10/9 5' AGTCGGATCCCGCCCCGCGCAGCCG-3'

    Following amplification in the PCR reaction a
DNA fragment is produced in which the flanking XbaI
site and the sequence identical to that present in
the corresponding region of clone 10/CT8-3 up to
the base immediately prior to the translation
initiator are faithfully reproduced except that a
novel BamHI site is introduced by the introduction
of the thymidine residue. This 930 bp fragment was
gel purified, and digested with XbaI and BamHI. It
was then cloned into pMS10-4 which had been
previously digested with XbaI and BamHI to yield
clone pMS10-5. In pMS10-5 the sequences required
for promoter activity associated with the MS10 gene
are reacted and modified such that the promoter can
now be fused to any gene via the BamHI site which
occurs immediately prior to the translation start
point. That these and no other modifications had
occurred was confirmed by sequence analysis.

EXAMPLE 9

Construction of a promoter fusion between Ms10 gene
and the glucuronidase reporter gene

5   The 1830 bp HindIII to BamHI fragment from
pMS10-5 (Figure 38) was ligated into pTAK1,
previously cut with HmdIII and Bam Hi. pTAK1 is
based on the binary plant transformation vector Bin
19 (Bevan, 1984; Nucleic Acids Research 12, 8711)
and carries the glucuronidase (GUS) reporter gene
10   and Nos 3' terminator (Figure 24). The resulting
plasmid was termed pMS10-6GUS and makes a
transcriptional gene fusion between the promoter of
the MS10 gene and the GUS reporter gene.

EXAMPLE 10

15   Transformation of tobacco plants with MS10
promoter gene constructs

         The recombinant vector pmS10-6GUS as mobilised
from E. Coli (TG-2) onto Agrobacterium tumefaciens
(LBA4404) in a triparental mating on L-plates with
20   E Coli (HB101) harbouring pRK2013. Transconjugants
were selected on minimal medium containing
kanamycin ($50\mu g/cm^3$) and streptomycin ($500\mu g/cm^3$).
         L-Broth (5 cm^3) containing kanamycin at 50
g/cm^3 was inoculated with a single Agrobacterium
25   colony. The culture was grown overnight at
30°C with shaking at 150 rpm. This culture (500μ1)
was inoculated into L-Broth containing kanamycin
(50μ g/cm^3) and grown as before. Immediately
before use the Agrobacteria were pelleted by
30   spinning at 3000 rpm for 5 minutes and suspended in
an equal volume of liquid Murashige and Skoog (MS)
medium.

         Feeder plates were prepared in 9 cm diameter
petri dishes as follows. Solid MS medium

supplemented with 6-benzyl-aminopurine (6-BAP) (1
mg/l) and 1-naphthaleneacetic acid (NAA) (0.1 mg/l)
was overlaid with Nicotiana tabacum var Samsun
suspension culture (1 cm^3). One 9 cm and one 7cm
filter paper discs were placed on the surface.

Whole leaves from tissue culture grown plants
were placed in the feeder plates. The plates were
sealed with "Nescofilm" (Trade Mark) and incubated
overnight in a plant growth room (26°C under bright
fluorescent light).

Leaves from the feeder plates were placed in
Agrobacteria suspension in 12 cm diameter petri
dishes and cut into 1- 1.5 cm^2 sections. After 20
minutes the leaf pieces were returned to the feeder
plates which were sealed and replaced in the growth
room. After 48 hours incubation in the growth room
the plant material was transferred to MS medium
supplemented with 6-BAP (1 mg/l), NAA (0.1 mg/l),
carbenicillin (500μg/cm^3) and kanamycin (100
μg/cm^3), in petri dishes. The petri dishes were
sealed and returned to the growth room.

Beginning three weeks after inoculation with
Agrobacterium, shoots were removed from the
explants and placed on MS medium supplemented with
carbenicillin (200 μg/cm^3) and kanamycin
(100μg/cm^3) for rooting. Transformed plants rooted
1-2 weeks after transfer.

Following rooting, transformed plants were
transferred to pots containing soil and grown in
the glasshouse. Roughly one month after transfer
the plants flowered.

The anthers of the tobacco plants containing
the pMS10-6GUS construct were sprayed for GUS
activity using standard procedures.

IV. DISRUPTER PROTEIN GENE

This module contains a gene, expressible in
plants, to inhibit mitochondrial function, hence
disrupting full expression of a selected plant
characteristic.

Accordingly we provide a method of inhibiting
gene expression in a target plant tissue comprising
stably transforming a plant cell of a type from
which a whole plant may be regenerated with a gene
construct carrying a tissue-specific or a
development-specific promoter which operates in the
cells of the target plant tissue and a disrupter
gene encoding a protein which is capable, when
expressed, of inhibiting respiration in the cells
of the said target tissue resulting in death of the
cells.

Preferably the disrupter gene is selected
from:

(a) The mammalian uncoupling protein (UCP) cloned
from mammalian (usually rat) brown adipose tissue.

(b) A mutated form of the gene for the β-subunit
of F_1-ATPase which has sequences added or deleted
such that these changes result in the retention of
the ability to assemble with other subunits but
interfere with function as an ATP synthase. The
ability of these altered subunits to assemble
correctly will be important as the required
phenotypic effect of their expression will depend
on their competition with wild-type subunits for
binding sites in the enzyme complex. Thus
complexes containing non-functional subunits will
only be weakly active and mitochondria harbouring
these complexes will be non-functional.

(c) A mutated, synthetic form of the oli 1 gene

encoding subunit 9 of the F_o-ATPase. Mutations created as described at (b) above.

(d) A mutated form of a mitochondrial transit pre-sequence which malfunctions during transfer
5   resulting, probably by blocking of receptor sites, in the disruption of protein transport to mitochondria.

(e) Gene constructs involving a fusion between the β-subunit gene from yeast and the β-glactosidase
10   gene from E. coli, resulting in expression of a disrupting fusion protein.

Preferably the promoter is a tapetum-specific promoter or a pollen-specific promoter, so that on expression of the said disrupter protein therein
15   the regenerated plant is in male sterile. More preferably the said tapetum-specific promoter has the sequence shown in Figure 17, 18, or 19 of the accompanying drawings.

The isolation and characterisation of these
20   gene control sequences of this invention are described in section III above.

This module, therefore, provides a method of preventing or inhibiting growth and development of plant cells based on gene constructs which inhibit
25   respiratory function. The technique has wide application in a number of crops where inhibition of particular cells or tissue is required.

Of particular interest is the inhibition of male fertility in maize for the production of F1
30   hybrids in situ. The concept of inhibition of mitochondrial function as a mechanism for male sterility arises from some previous research on T-type cytoplasmic male sterility in maize (cms-T) which has shown an association between the male

sterile phenotype and mitochondrial dysfuction.
Although a direct causal relationship has yet to be
established between mitochondrial dysfunction and
cms-T, an increasing body of evidence suggests that

5 fully functional mitochondria, particularly in the
tapetal cells, are essential. This is particularly
critical during microsporogenesis since the
metabolic demands placed on the tapetal cells
results in a 40-fold increase in mitochondrial

10 number.

Thus we provide a number of negative mutations
which act upon mitochondria to uncouple oxidative
phosphorylation. When specifically expressed in
maize anther tissue these mutations will result in

15 a male sterile phenotype.

The proposed disrupter protein, UCP, is
instrumental in the thermogenesis of mammalian
brown adipose tissue and exists as a dimer in the
mitochondrial inner membrane forming a proton

20 channel and thus uncoupling oxidative
phosphorylation by dissipation of the proton
electrochemical potential differences across the
membrane.

An alternative is the use of chimeric gene

25 constructs in which domains are swapped, creating
non-functional proteins. The target proteins here
are the β-subunit of F_1-ATPase and subunit 9 of the
F_0- ATPase. During assembly of functional ATPase
complexes, the altered chimeric subunits will

30 complete for binding sites normally occupied by the
naturally occurring subunits, particularly when the
chimeras are over expressed compared with the
endogenous genes. Mitochondrial function will be
disrupted since F_1 and F_0 ATPase's assembled with

altered subunits are likely to be weakly active or
non-functional.

The method employed for transformation of the
plant cells is not especially germane to this
invention and any method suitable for the target
plant may be employed. Transgenic plants are
obtained by regeneration from the transformed
cells. Numerous transformation procedures are
known from the literature such as agroinfection
using Agrobacterium tumefaciens or its Ti plasmid,
electroporation, microinjection of plant cells and
protoplasts, microprojectile transformation and
pollen tube transformation, to mention but a few.
Reference may be made to the literature for full
details of the known methods.

The development and testing of these gene
constructs as disrupters of mitochondrial function
in the unicellular organism, yeast, will now be
described. A mechanism by which these gene
constructs may be used to inhibit plant cell growth
and differentiation in transformed plants will also
be described. The object of these procedures is to
use yeast as a model system for the identification
and optimisation of gene constructs for expressing
proteins which disrupt mitochondrial function.
Plant cells will then be transformed with the
selected constructs and whole plants regenerated
therefrom.

Specific embodiments of the module will now be
illustrated by the following Example.

EXAMPLE

It was known from reports in the literature
that the rat UCP gene inserted in the yeast/E. coli
shuttle vector gave only low levels of expression

of UCP. The yeast was <u>Saccharomyces cerevisiae</u>
strain YM147 and the UCP gene was available on
plasmid pCGS110-UCP.

Given the lack of useful expression levels
5   with the wild type gene, modification of the rat
UCP gene using site directed mutagenesis was
carried out. the following modifications were
made:

1.   Introduction of a BamHI site seven nucleotides
10   5' to the AUG methionine initiation codon;

2.   Modification of the sequence around the AUG
methionine initiation codon to conform to the yeast
consensus sequence ATAATG;

3.   Deletion of an internal BamHI site; and,
15   4.   Introduction of a BamHI site one nucleotide 3'
to the TAG termination codon.

These modifications result in the deletion of
the untranslated 5' and 3' rat UCP sequences as
well as the introduction of a yeast consensus
20   sequence at the methionine initiation codon.

The 1.9 kb EcoRI/PstI fragment from the
plasmid pCGS110-UCP (a map of the plasmid is shown
in Figure 26 and the mRNA sequence of the UCP gene
is shown in Figure 27) carrying the GAL10 promoter
25   region and the rat UCP cDNA was cloned into the
EcoRI/PstI sites of M13mp19 DNA. Sequencing of the
resultant construct was carried out to ensure the
correct structure.

Site directed mutagenesis was carried out
30   according to the directions given in the Amersham
(Trade Mark) mutagenesis kit using three different
oligonucleotides as follows:

UCP-1 wild type	CTCTGCCCTCCGAGCCAAGATGGTGAGTT
mutant	CTCTGCCCTCGGATCC(ATAATG)GTGAGTT
UCP-2 wild type	TGCGACTCGGATCCTGGAACG
mutant	TGCGACTCGGTTCCTGGAACG
UCP-3 wild type	ACCACATAGGCGACTTGGAG
mutant	ACCACATAGGATCCGACTTGGAG

Oligonucleotide UCP-1 was used to introduce the yeast consensus sequence (bracketed) which occurs around the methionine initiation codon, as well as the introduction of the BamHI cleavage site (underlined).

Oligonucleotide UCP-2 was used to delete an internal BamHI site (underlined).

Oligonucleotide UCP-3 was used to introduce a BamHI site immediately after the TAG stop codon (underlined).

These three mutations allowed the isolation of the entire UCP coding sequence on a 0.93 kb BamHI fragment.

After selection of mutant clones the modified DNA was digested with BamHI. Three clones from twenty selected gave inserts of 0.93 kb upon digestion with BamHI.

Sequencing of the clones UCPS1 and UCPS4 revealed that the UCP gene had been correctly modified with no unwanted changes present. The UCP gene was then transferred to the yeast expression plasmid pKV49 which allows expression of foreign genes in S. cerevisiae under the control of the strong PGK promoter and the GAL1-10 UAS allowing induction/repression of the foreign gene according to whether or not galactose is present in the growth medium. The 0.93 kb BamHI fragment

containing the modified UCP gene was cloned into
pKV49 at the BglII restriction site, resulting in
the construct pKV49-UCP.

TRANSFORMATION OF YEAST WITH pKV49-UCP CONSTRUCT

5   a) Development Of Suitable Yeast Strain

For a recipient for the pKV49-UCP construct we
needed a yeast strain carrying the appropriate
markers for transformation and allowing induction
of gene expression from the GAL1-10 UAS while being

10   unable to utilise glactose as a carbon and energy
source (GAL1, GAL2).   Such strains were generated
by mating yeast strains YM147 and SF747.   After
selection of diploids on minimal plates containing
uracil, the colonies were transferred to

15   sporulating media.   The resulting spores were grown
on YDP plates prior to the resulting yeast colonies
being characterised (Figure 28).   Two new yeast
strains BET9 (ura3, trpll, leu2, his3, gall) and
BE27 (ura3, trp1, leu2, gall) were isolated, both

20   of which are suitable for transformation with PkV49
based constructs.

b) Yeast Transformation

Yeast strains BET9 and BE27 were transformed
with pKV9 and pKV49-UCP DNA; transformants were

25   selected using the appropriate auxotrophic
selection (leu) and checked by plasmid isolation
followed by restriction mapping.   Single colonies
from each of the four different transformants
BET9/pKV49, BET9/pKV49-UCP, BE27/pKV49 and

30   BE27/pKV49-UCP) were resuspended in sterile water
prior to being spotted onto plating media
containing a variety of carbon sources (Figure 29)
both in the presence and absence of galactose.
Results from these plate tests (Figure 29)

indicated that on a few of the carbon sources used, the presence of both galactose and the pKV49-UCP construct resulted in poorer growth of the resulting yeast colonies. The greater effect on retardation of growth was observed with the glycerol/casamino (gly/cas) medium containing glactose for both pKV49-UCP transformants. Transformants either lacking the UCP gene or induced by galactose grew at the same rate as the untransformed BET9 and BE27 strains.

GROWTH CURVE ANALYSIS

As plating tests had indicated poor growth of pKV49-UCP transformants on gly/cas medium in the presence of galactose, growth curve analysis in liquid culture was carried out to determine more accurately the magnitude of the growth defect. The results in Figure 30 substitute the results of plating tests and indicate that neither the presence of pKV49-UCP DNA or galactose alone is sufficient to have any effect on the yeast cell growth rates, while the presence of both severely retards growth. As our initial results using the yeast strain YM147 transformed with the construct pCGS110-UCP had not shown any significant growth defect on any of the tested carbon sources in the presence of galactose, it would appear that the modification of the UCP gene and/or the use of a different vector (pKV49) have resulted in an observable growth defect.

ANALYSIS OF UCP EXPRESSION

As the growth curve analysis had indicated no detectable differences between the BE27 and BET9 transformants (Figure 30), it was decided only to use the BET9 transformants in subsequent

experiments. Repeat growth analysis on gly/cas
medium both in the presence and absence of
galactose was carried out with the BET9
transformants. Cultures were allowed to grow for

5   47 hours to ensure that the same growth curve
characteristics observed previously (Figure 31)
were repeated. Cells were then harvested, total
cell proteins were isolated and fractionated (in
duplicate) by SDS-PAGE on a 10% polyacrylamide gel.

10   One set of fractionated proteins were stained with
Coomassie Blue to ensure equal loading of the
proteins while the other set were transferred to
nylon membrane and subjected to Western blot
analysis using the rat UCP antibody. The Western

15   blot showed two main features:
1)   The comparative level of UCP expression
between the   BET9/pKV49-UCP transformant and
the   VY147/pCGS110-UCP transformant reveals that
the UCP   expression has increased approximately

20   50-100 fold as a consequence of our modifications.
2)   The yeast transformant which exhibits
defective growth when grown on gly/cas medium in
the presence of galactose also expresses
substantial amounts of UCP.

25   It can be concluded from these results that
the modification of the UCP gene and/or its
subsequent cloning into the pKV49 vector has
resulted in the increased level of UCP expression

30   relative to the levels initially detected with the
pCGS110-UCP construct. Growth curve analysis
indicates that the expression of UCP has an effect
on the growth rates of yeast cells grown under
certain conditions. As yet we have not been able

to identify the specific effect that the increased
levels of UCP expression have on yeast cell growth
rates but preliminary results implicate a
mitochondrial defect.

5   Growth curve analysis carried out in the
raffinose medium (a fermentable carbon source which
should not affect Gal regulation) of the BET9
transformants grown in the presence or absence of
galactose indicate that the presence of both the
10   UCP gene and galactose has no effect on growth
rates (Figure 32). Western blot analysis of the
proteins isolated from cells harvested during these
growth curves reveals levels of UCP expression
similar to those found in cells grown in gly/cas
15   medium in the presence of galactose.

The UCP detected in BET9/pKV49-UCP
transformants grown without added galactose is
probably due to galactose residues released into
the medium by hydrolysis of raffinose, possibly
20   during the autoclaving. These observations
indicate that the presence of UCP in yeast cells
grown on a fermentable carbon source (no
requirement for oxidative phosphorylation) has no
effect on cell growth rates, while cells growing on
25   the gly/cas medium (a non-fermentable carbon
source) expressing UCP exhibit defective growth.

LOCATION OF UCP IN YEAST CELLS

Rat UCP is a major component of the
mitochondrial inner membrane of brown adipose
30   tissue. Unlike many other polypeptides found in
the inner membrane it does not contain a cleavable
signal sequence, the targeting information being
encoded internally within the amino acid sequence
of the protein. As our results indicate that the

expressed UCP has an effect on the rate of yeast
cell growth then it is important to determine the
precise location of the protein expressed in yeast
cells.   Initial Western blot analysis of total
mitochondrial proteins shows the UCP expressed by
the pKV49-UCP transformant to be located in the
mitochondrial fraction.

Subsequent mitochondrial fractionation
revealed that the majority of the UCP is located in
the inner membrane fraction of yeast mitochondria.
Although some of the UCP appears to be located in
the inner-membrane space, this observation is most
likely due to contamination of this fraction with
some of the inner membrane fraction.   Similar
results have been obtained with the location of the
β-subunit of the F_1-ATPase complex from yeast cell
mitochondria.   The β-subunit which is a component
of the inner membrane is also detected in our
inter-membrane space preparations.   However, these
results do show that the targeting information
within the rat UCP is sufficient to target the UCP
to the inner membrane of yeast mitochondria where
it could function a an uncoupler protein.

UCP TRANSCRIPT ANALYSIS

RNA has been isolated from many of the growth
curve experiments described previously.   We are
currently carrying out Northern blot analysis in
order to determine whether the patterns of UCP
expression are reflected by the UCP transcript
signals

EFFECT OF COPY NUMBER ON UCP EXPRESSION

The transformation of yeast cells with shuttle
vectors containing the origin of replication from
the yeast 2 μm circle, such as pKV49-UCP, results

in these plasmids being present at approximately 40-50 copies per cell. Consequently any foreign gene carried by the plasmid will be present at the same relatively high copy number which may result in the expression of the foreign protein at a higher level than would be seen for a gene present at a low copy number. We have therefore attempted to lower the copy number of the UCP gene by integrating it into the yeast chromosome at a single site resulting in a genetically stable, single-copy transformant. The vector YIp5 (Figure 33) is an integrating yeast vector carrying the ura3 gene; it is unable to replicate autonomously in yeast.

The 1.8 kb EcoRI/SalI fragment from pKV49-UCP containing the rat UCP gene along with the PGK promoter and GAL UAS was cloned into the EcoRI/SALI sites of YIp5 DNA. The resultant plasmid UIP-UCP (Figure 33) was checked by restriction enzyme mapping to ensure the UCP gene was correctly inserted . The YIP-UCP plasmid was cut with the restriction enzyme EcoRV (which cuts in the middle of the URA3 gene (Figure 33) and the linearised YIP/UCP DNA was used to transform the yeast cell lines BET9 and BE27. Transformants were initially screened on minimal plates by selecting for uracil prototrophy and after 7-10 days two transformants from each cell line were streaked out onto YPD plates (non-selective). The transformants were then subjected to four consecutive periods of growth on non-selective medium. One hundred colonies from each of the original four transformants were then replica plated onto both non-selective (YDP) and selective media (minimal

plants + ura). All colonies grew on the selective
media indicating that the URA3 gene, which is
genetically linked to the UCP gene (Figure 33), had
been integrated to the yeast cell chromosome.

5   Chromosomal DNA was isolated from each of the four
transformants, digested with the restriction enzyme
EcoRI and fractionated on a 0.08% agarose gel.
Southern blot analysis using a labelled UCP probe
indicated that the UCP gene is present in the yeast

10   chromosome of all four transformants. Western blot
analysis using the rat UCP antibody will show the
level of UCP expression in these transformants.
Growth curve analysis of these transformants grown
in the presence of galactose shows that they may

15   have growth inhibition consistent with a
mitochondrial defect.

EXAMPLE 3

MODIFICATION OF THE β-SUBUNIT OF F_1ATPASE

        The second approach we have taken to

20   introducing mutations affecting mitochondrial
function is the directed modification of functional
mitochondrial proteins which when expressed in
yeast might be expected to interfere with the
generation of ATP. The protein chosen for this

25   approach is the β-subunit of the F_1-ATPase complex.
The DNA sequence of the yeast β-subunit gene is
known and the gene has been independently cloned
and sequenced in our laboratory 918).

        The F_1ATPase portion of ATP synthase catalyses

30   the terminal step of oxidative phosphorylation F_1
is an assembly of five different polypeptides
designated α, β, γ, δ and ε. Experiments carried
out by Parsonage et al on modification of the
β-subunit of F_1-ATPase from E. coli identified

specific amino acid residues of the β-subunit that
appear to be very important for catalysis of both
ATP synthesis and hydrolysis. Two mutations in
particular were shown to result in greatly impaired

5   catalysis without causing major structural
perturbation of the F_1-ATPase. One of these
mutations resulted from changing the strongly
conserved lysine residue occurring in the catalytic
nucleotide-binding domain at position 155 to a

10   glutamine residue while the other mutation resulted
from changing the methionine residue at position
209 to a leucine residue. Both of these mutations
have been reposed to exert their effect by the
prevention of confirmational changes required from

15   the catalytic cooperativity in the F_1 complex.

        As the assembly of these mutated β-subunit
proteins into the F_1-ATPase is not affected, then
it was felt that similar mutants of the β-subunit
in yeast might compete for assembly into F_1-ATPase.

20   It was thought that the result of having both
wild-type and mutated β-subunits in the same
F_1-ATPase would perhaps result in impaired
catalysis resulting in a decrease in ATP production
and retarded cell growth.

25   The β-subunit of F_1-ATPase from a wide
variety of sources has been shown to by highly
conserved at the amino acid sequence and comparison
of the S cerevisiae β-subunit amino acid sequence
with that from E. Coli confirms that the lysine and

30   methionine residues shown by Parsonage et al to be
very important for catalytic activity are
conserved, with the lysine and methionine residues
occurring at positions 196 and 255 respectively on
the yeast β-subunit sequence.

In order to carry out SDM the wild-type β-subunit gene from yeast was isolated from the plasmid pGR208 (Figure 34) as an EcoRI/BamHI fragment which was cloned into M13mp19. Two

5   mutated β-subunit genes were constructed: mutant BB1 has both the Met255 and Lys196 converted to isoleucine and glutamine respectively while mutant BB2 has only the lysine to glutamine mutation (Figure 34). Following sequence analysis to ensure

10   correct mutagenesis with no unwanted mutations, the mutated β-subunit genes were removed from mp19 by EcoRI/BamHI digests. The fragments containing the genes were then blunt-ended and ligated to BglII digested pKV49 (Figure 35) which had previously

15   been blunt-ended. We have both mutated β-subunit genes cloned into pKV49 (pKV49-BB1 and pKV49-BB2) and have transformed the yeast strain BET9 with both these constructs. Growth curve and plate growth from both mutated β-subunit transformants

20   show tht the transformants have altered growth characteristics which are consistent with a mitochondrial defect.

Concurrently with the transformation of strain BET9 with the mutated β-subunit genes, gene

25   disruption may be used to construct a derivative of strain BET9 which will fail to synthesize β-subunit. The resultant strain will therefore be unable to grow on non-fermentable carbon sources although it will be easily maintainable on a

30   fermentable carbon source such as glucose. Transformation of this strain with plasmids bearing the mutated β-subunit genes, followed by measuring the transformants' growth characteristics on a non-fermentable carbon source, shows that the

altered β-subunit is unable to support oxidative
phosphorylation.

EXAMPLE 4

FUSION PROTEINS

5   An alternative strategy for selectively
perturbing mitochondrial function is the expression
of a fusion protein which results in either poor or
no yeast cell growth. The candidate fusion protein
chosen from this project contains the N-terminal
10   region of the yeast ATP synthase β-subunit fused to
most of β-galactosidase from E. coli and has been
constructed by gene fusion (Figure 36). This
β-subunit/β-galactosidase fusion protein has
already been shown to be targeted to the inner
15   membrane of yeast mitochondria 921) and cells
expressing this fusion protein appear to be unable
to grow on a non-fermentable carbon source. In the
presence of the fusion protein the transducing
capacity of the mitochondrial membrane as measured
20   by the ^{32}P-ATP exchange reaction is only 9% of that
measured in the absence of the fusion protein. As
yet the mechanism of this description has not been
evaluated but the gene fusion is thought to produce
a protein which becomes trapped in the inner
25   membrane and interferes with function(s) essential
for respiratory growth.

CONSTRUCTION OF THE ATP2/LacZ GENE FUSION

          The plasmid pGR208, which contains the yeast
ATP2 DNA encoding ATP synthase β-subunit gene
30   (Figure 34), was digested with EcoRI plus BamHI
resulting in the release of a 1.1kb fragment coding
for the first 350 amino acids of the β-subunit
protein. pMUR1720 is a pUC8 based plasmid which
contains a LacZ gene contained within an EcoRI/NarI

fragment (Figure 36). Cloning of the 1.1kb
EcoRI/BamHI DNA fragment coding for the first 350
amino acids of the yeast β-subunit protein into the
EcoRI/BamHI sites of pMUR1720 (Figure 36A) results

5   in an in-frame fusion between the 350 amino acids
of the β-subunit and the entire (minus the first
eight amino acids) LaZ protein (Figure 36). The
entire β-subunit/LacZ gene fusion is now contained
on the 4.3kb EcoRI/NarI fragment in construct

10   pMUR1720-BLZ (Figure 36). This 4.3kb EcoRI/NarI
fragment is currently being cloned into the pKV49
vector resulting in the pKV49-BLZ construct (Figure
37) which can be used to transform the yeast
strains BET9 and BE27 and show that when induced by

15   galactose growth defects consistent with
mitochondrial inhibition arise.

EXAMPLE 5
Construction of a promoter fusion between the MS10
gene and the UCP gene

20   The 1830 bp HindIII to BamH1 fragment from
pMS10 was ligated into the binary plant
transformation vector Bin19 previously cut with
HindIII and BamH1.

        Following ligation the resultant plasmid was

25   cut with BamH1 and ligated to the 930 bp UCP BamH1
fragment from plasmid pUC/UCP (s derivative of
pUC19 contyaininbg the modified UCP gene cloned at
the BamH1 site) to construct a fusion between the
MS10 gene promoter and the UCP gene. Finally the

30   nos 3' terminator obtained as a 250 bp Sst1- EcoR1
fragment from vector pTAK1 wasligated into the
MS10-UCP construct previouslt cut with Sst1 and
EcoR1.

The resulting plasmid is termed pBin/MS10-UCP
(Figure 39) and contains the MS10 promoter, the UCP
gene, nos 3' terminator expression cassette located
between the right and left border sequences of
5   Agrobacterium T-DNA allowing efficient
transformation into tobacco cells.

EXAMPLE 6

Transformation of tobacco plants with pBin/MS10
promoter-gene-constructs

10   The recombinant vector pBin/MS10-UCP was
mobilised from E Coli (TG-2) onto Agrobacterium
tumefaciens (LBA4404) in a triparental mating on
L-plates with E Coli (HB101) harbouring pRK2013.
Transconjugants were selected on minimal medium
15   containing kanamycin ($50\mu g/cm^3$) and streptomycin
($500\mu g/cm^3$).

L-Broth (5 cm^3) containing kanamycin at 50
g/cm^3 was inoculated with a single Agrobacterium
colony. The culture was grown overnight at 30°c
20   with shaking at 150 rpm. This culture ($500\mu 1$) was
inoculated into L-Broth containing kanamycin (50μ
g/cm^3) and grown as before. Immediately before use
the Agrobacteria were pelleted by spinning at 3000
rpm for 5 minutes and suspended in an equal volume
25   of liquid Murashige and Skoog (MS) medium.

Feeder plates were prepared in 9 cm diameter
petri dishes as follows. Solid MS medium
supplemented with 6-benzyl-aminopurine (6-BAP) (1
mg/l) and 1-naphthaleneacetic acid (NAA) (0.1 mg/l)
30   was overlaid with Nicotiana tabacum var Samsun
suspension culture (1 cm^3). One 9 cm and one 7cm
filter paper discs were placed on the surface.

Whole leaves from tissue culture grown plants
were placed in the feeder plates. The plates were

sealed with "Nescofilm" (Trade Mark) and incubated
overnight in a plant growth room (26°C under bright
fluorescent light).

Leaves from the feeder plates were placed in
5   Agrobacteria suspension in 12 cm diameter petri
dishes and cut into 1- 1.5 cm^2 sections. After 20
minutes the leaf pieces were returned to the feeder
plates which were sealed and replaced in the growth
room. After 48 hours incubation in the growth room
10   the plant material was transferred to MS medium
supplemented with 6-BAP (1 mg/1), NAA (0.1 mg/1),
carbenicillin (500μg/cm^3) and kanamycin (100
μg/cm^3), in petri dishes. the petri dishes were
sealed and returned to the growth room.

15   Beginning three weeks after inoculation with
Agrobacterium, shoots were removed from the
explants and placed on MS medium supplemented with
carbenicillin (200 μg/cm^3) and kanamycin
(100μg/cm^3) for rooting. Transformed plants rooted
20   1-2 weeks after transfer.

Following rooting, transformed plants were
transferred to pots containing soil and grown in
the glasshouse. Roughly one month after transfer
the plants flowered.

25   The anthers of the tobacco plants containing
the pBin/MS10-UCP construct were were assayed for
expression of the UCP gene by Northern blotting of
RNA samples, and the effect of UCP expression on
pollen development determined.

30

1.   A plant gene construct comprising a
disrupter gene encoding a protein which is
capable of disrupting the biogenesis of viable
pollen, and a gene regulatory sequence which
5   includes a promoter sequence inducible by
external application of an exogenous chemical
inducer to a plant containing the construct.

2.   A plant gene construct as claimed in claim
1 in which the regulatory sequence includes an
operator sequence controlling said disrupter
gene and a repressor gene encoding a repressor
5   protein adapted to interact with the disrupter
gene operator and inhibit expression of the
dirupter protein, said repressor protein gene
being regulated by the chemically inducible
promoter.

3.   A plant gene construct as claimed in claim
1 or claim 2 in which the construct also
includes a male flower specific promoter
sequence operatively linked to said disrupter
5   protein gene, for restricting expression of
the disrupter protein gene to male flower
parts of a plant.

4.   A plant gene construct as claimed in claim
          1 or 2 or 3 in which the construct comprises:

          (a) a first gene promoter sequence responsive
          to the presence or absence of an exogenous
5   chemical inducer,

          (b) a gene encoding a repressor protein under
          control of the said first promoter sequence;

          (c) an operator sequence responsive to the
          said repressor protein expressed by the
10   repressor protein gene;

          (d) a male flower specific gene promoter
          sequence expressible only in male parts of a
          plant; and,

          (e) a gene encoding a disrupter protein
15   capable of disrupting biogenesis of viable
          pollen;

          whereby the presence or absence of the
          exogenous chemical inducer in the plant
          enables selection of male fertility or
20   sterility.

5.   A plant gene construct as claimed in any
          of claims 1 to 4 in which the chemically
          inducible gene promoter sequence is a 27kd
          subunit isolated from the maize glutathione-S-
5   transferase (GST II) gene.

6.   A plant gene construct as claimed in claim
5, in which the inducer for the chemically
inducible gene promoter is N,N-diallyl-2,2-
dichloro-acetamide or benzyl-2-chloro-4-(tri-
fluoro- methyl)-5-thiazole-carboxylate.

7.   A plant gene construct as claimed in claim
6 in which the inducer is N,N-diallyl-2,2-di-
chloro-acetamide.

8.   A plant gene construct as claimed in any
of claims 1 to 7 in which the disrupter gene
is the mammalian uncoupling protein (UCP)
gene.

9.   A gene construct as claimed in any of
claims 1 to 7, in which the disrupter gene is
a mutated form of the gene for the β-subunit
of F_1-ATPase which has sequences added or
deleted such that these changes result in the
retention of the ability to assemble with
other subunits but interfere with function as
an ATP synthase.

10.   A plant gene construct as claimed in any
of claims 1 to 7, in which the disrupter gene
is a mutated, synthetic form of the oli 1 gene
encoding subunit 9 of the F_o-ATPase.

11.   A plant gene construct as claimed in any
of claims 1 to 7, in which the disrupter gene
is a mutated form of a mitochondrial transit
pre-sequence which malfunctions during
5   transfer resulting in the disruption of
protein transport to mitochondria.

12.   A plant gene construct as claimed in any
of claims 1 to 7 in which, the disrupter gene
is a gene construct carrying a fusion between
the β-subunit gene from yeast and the
5   β-glactosidase gene from <u>E. coli</u>, resulting in
expression of a disrupting fusion protein.

13.   A plant gene construct as claimed in any
of claims 2 to 12, in which the repressor gene
is a DNA-binding protein gene place under
control of the chemically inducible promoter,
5   the encoded DNA-binding protein gene being
capable of interaction with the operator
sequence associated with the disrupter gene.

14.   A plant gene construct as claimed in claim
13, in which the DNA-binding protein gene
encodes a repressor protein so that on
expression of the DNA-binding protein gene
5   expression of protein by the disrupter gene is
inhibited.

15.   A plant gene construct as claimed in claim
12 or 13, in which both the DNA-binding
protein gene and the said operator are of
non-plant origin.

16.   A plant gene construct as claimed in claim 15,
in which both the DNA-binding protein gene and
the said operator are of bacterial origin.

17.   A plant gene construct as claimed in claim
16, in which the repressor gene and the
operator are derived from the lacI gene of
Escherichia coli.

18.   A plant gene construct as claimed in claim
17, in which the repressor and operator are
isolated from the plasmid, designated
p35SlacI, deposited in an Escherichia coli,
strain TG-2, host with the National Collection
of Industrial and Marine Bacteria Limited,
Aberdeen, United Kingdom, on 12th December
1988, under the Accession Number NCIB 40092.

5

19.   A plant gene construct as claimed in any
of claims 2 to 12 in which the disrupter gene
operator is a pseudo-operator sequence, and
the repressor gene is a mutant regulatory gene
encoding a repressor having an amino acid
sequence which binds to the pseudo-operator.

5

20.   A plant gene construct as claimed in any
of claims 3 to 19 in which the male flower
specific sequence is the polynucleotide shown
in Figure 15 herewith, which is specifically
expressed in male flower tissue or a variant
5   thereof permitted by the degeneracy of the
genetic code.

21.   A plant gene construct as claimed in claim
20, in which the male flower specific sequence
is isolated from plasmid pMS10 which has been
deposited in an Escherichia coli strain R1
5   host with the National Collection of
Industrial & Marine Bacteria on 9th January
1989 under the Accession Number NCIB 40090.

22.   A plant gene construct as claimed in claim
21 or 22 in which the male flower specific
and disrupter gene are isolated from the
plasmid designated pBin/MS10-UCP having the
5   structure shown in Figure 39 of the
accompanying drawings.

23.   A plant gene construct as claimed in any
of claims 3 to 19, in which the male flower
specific sequence comprises the polynucleotide
shown in Figure 16 herewith, which is
5   specifically expressed in male flower tissue
or a variant thereof as permitted by the
degeneracy of the genetic code.

24.   A plant gene construct as claomed in claim
23, in which the male flower specific gene is
isolated from the plasmid pMS14 which has been
deposited in an <u>Escherichia coli</u> strain DH5α
5   host, with the National Collection of
Industrial & Marine Bacteria on 9th January
1989 under the Accession Number NCIB 40099.

25.   A plant gene construct as claimed in any
of claims 3 to 19, in which the male flower
specific sequence comprises the polynucleotide
shown in Figure 17 herewith, which is
5   specifically expressed in male flower tissue
or a variant thereof permitted by the
degeneracy of the genetic code.

26.   A plant gene construct as claimed in claim
25, in which the male flower specific sequence
is isolated from the plasmid pMS18 which has
been deposited in an <u>Escherichia coli</u> strain
5   R1 host, with the National Collection of
Industrial & Marine Bacteria on 9th January
1989 under the Accession Number NCIB 40100.

27.   A transformed plant and plant parts such
as cells, protoplasts and seeds incorporating
the construct calimed in any of the preceding
claims.

28.   A plant which is restorably male sterile,
in which said plant contains, stably
incorporated in its genome by transformation,
the recombinant plant gene construct claimed
5   in any of the preceding claims.

29.   A plant or plant part as claimed in claim
27 or 28, in which the plant is
dicotyledonous.

30.   A plant or plant part as claimed in claim
27 or 28, in which the plant is
monocotyledonous.

RESTORABLE MALE STERILITY

MINUS CHEMICAL — disrupter protein moce
ALL PLANTS ARE MALE STERILE
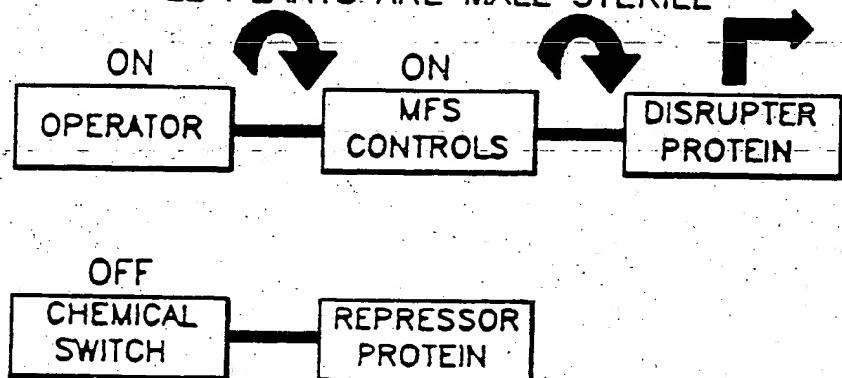
FIG. 1.

PLUS CHEMICAL — no disrupter protein
ALL PLANTS ARE FERTILE
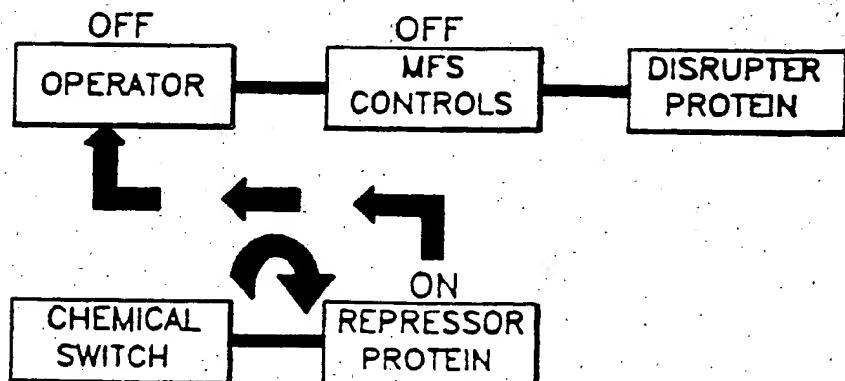
FIG. 2.

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FIG. 3.

Stimulation of GST activity in roots and shoots by safener treatment

23 hrs

44hrs
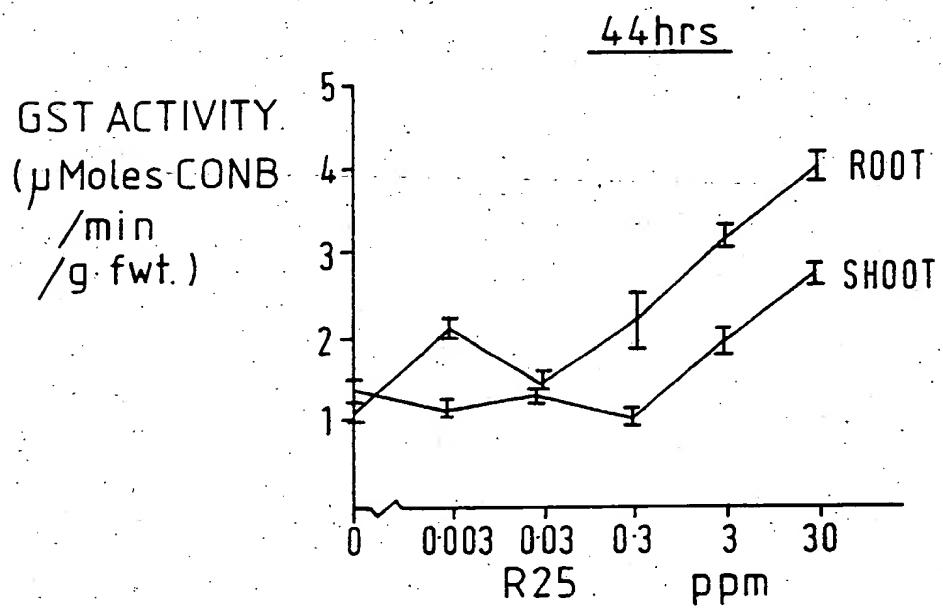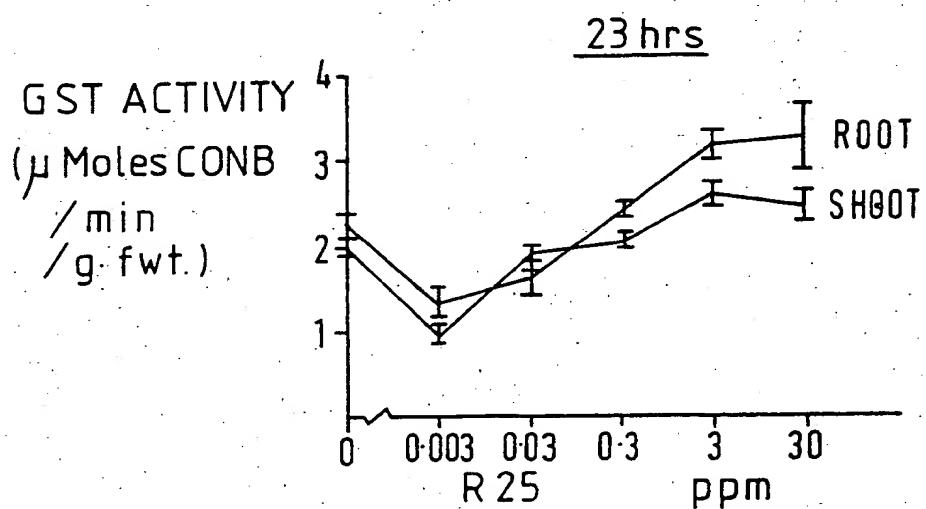
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FIG. 4.

Separation of GST I and GST II in roots tissues treated with R25

GST FROM MAIZE ROOTS TREATED WITH R25

(≃ 6 ppm)
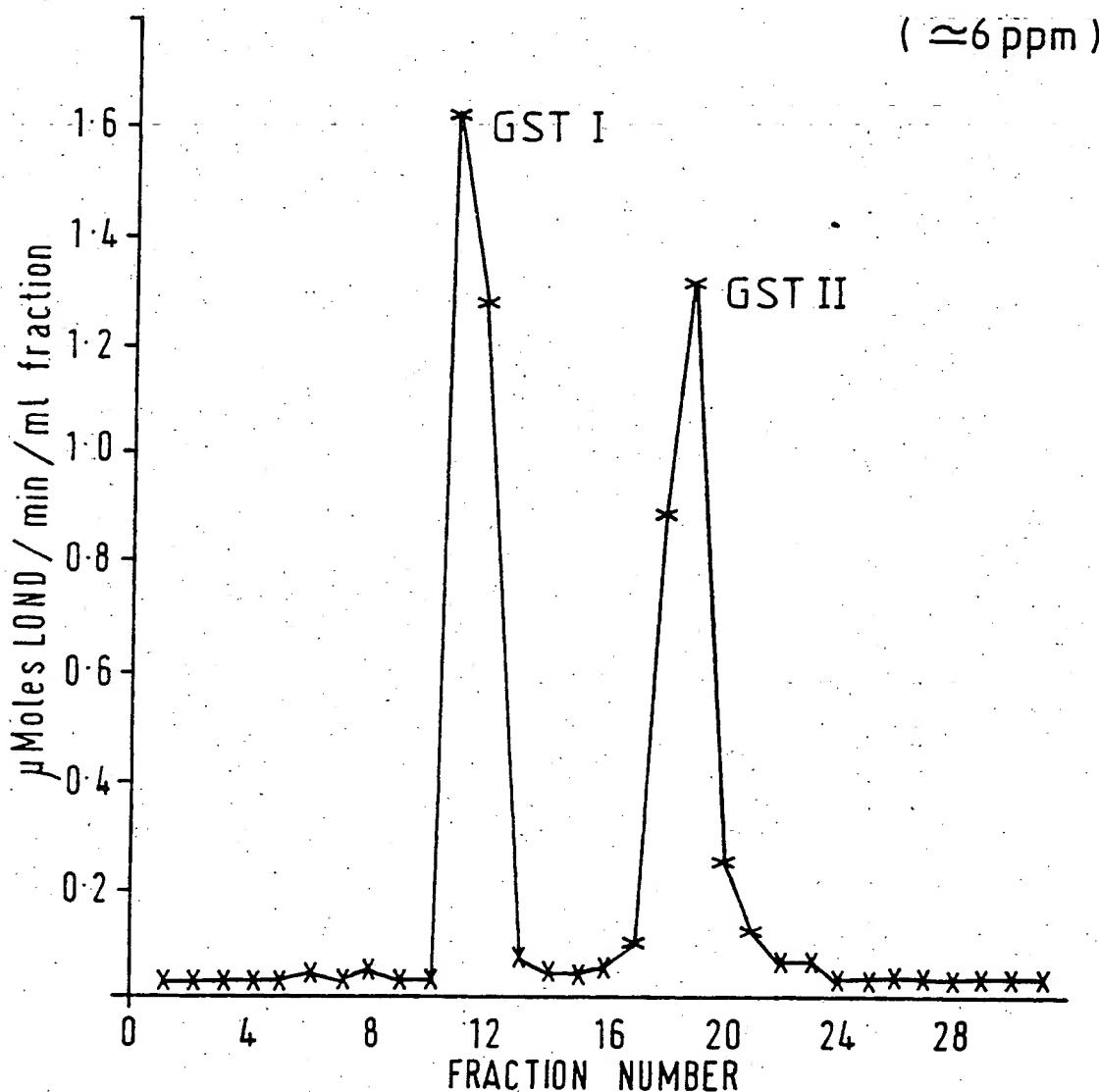
GST I ≃ 0.9% OF TOTAL SOL PROTFIN

GST II ≃ 2.5% OF TOTAL SOL PROTFIN

[USING DATA FROM MOZER (MONSANTO)]

SUBSTITUTE SHEET

FIG. 5.

GST I is the only isoform present in untreated anthers

GST FROM UNTREATED MAIZE ANTHERS

0.7% OF TOT SOL PROTEIN IN CRUDE EXTRACT

μMoles/min/ml fraction

FRACTION NUMBER
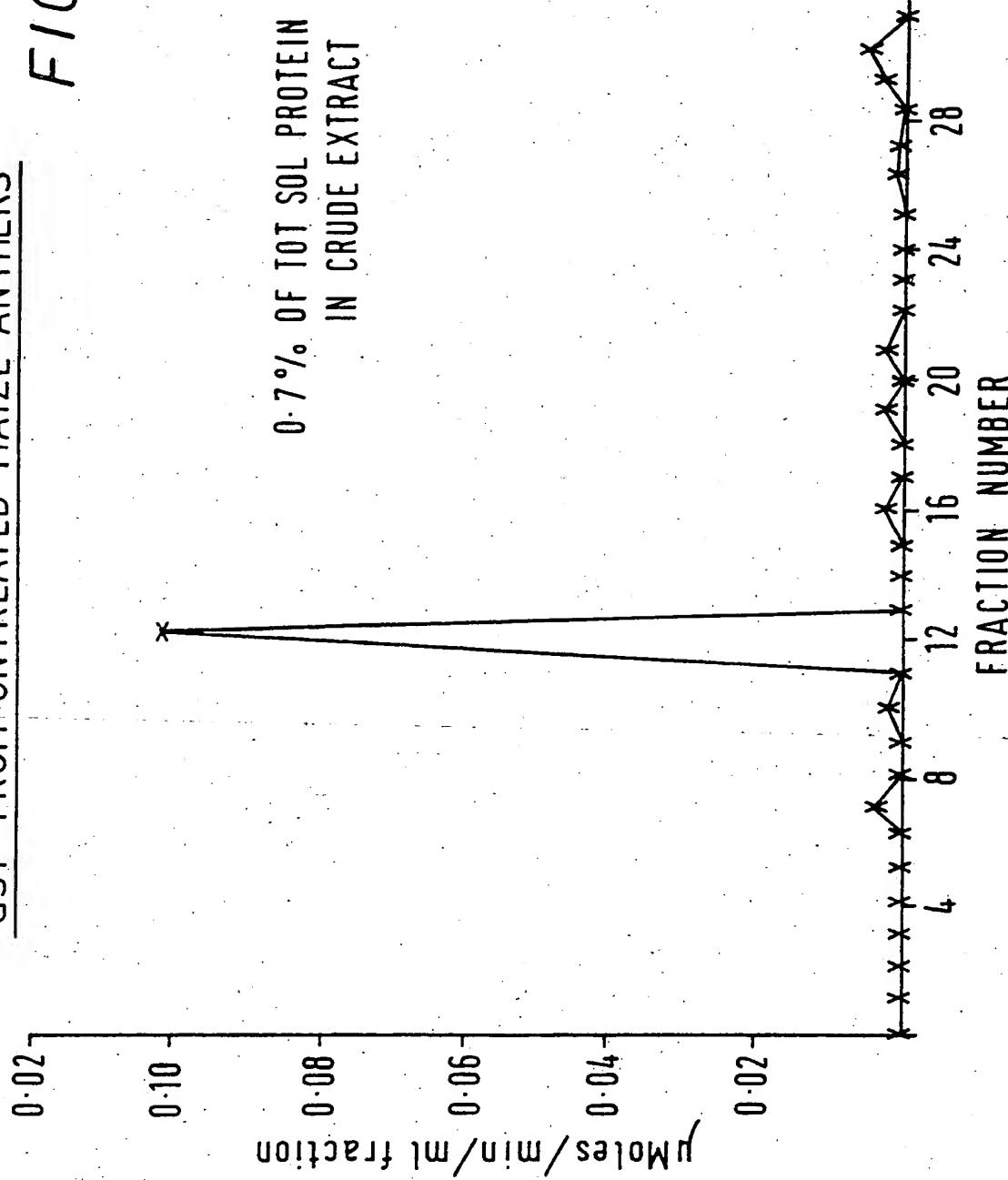
GST II activity is stimulated in anthers treated with R25

GST ISOFORM SEPARATION
R25 Injection 69Hrs

—— Activity
—— Gradient

768 μg R25 INJECTED

177 ppm R25 IN TASSEL BRANCHES

FIG.6.

GST I

87.4%
C.22% tot sol prot

GST III 2.6%
0.09% tot sol prot

Fraction Number

μMoles/min/ml fraction
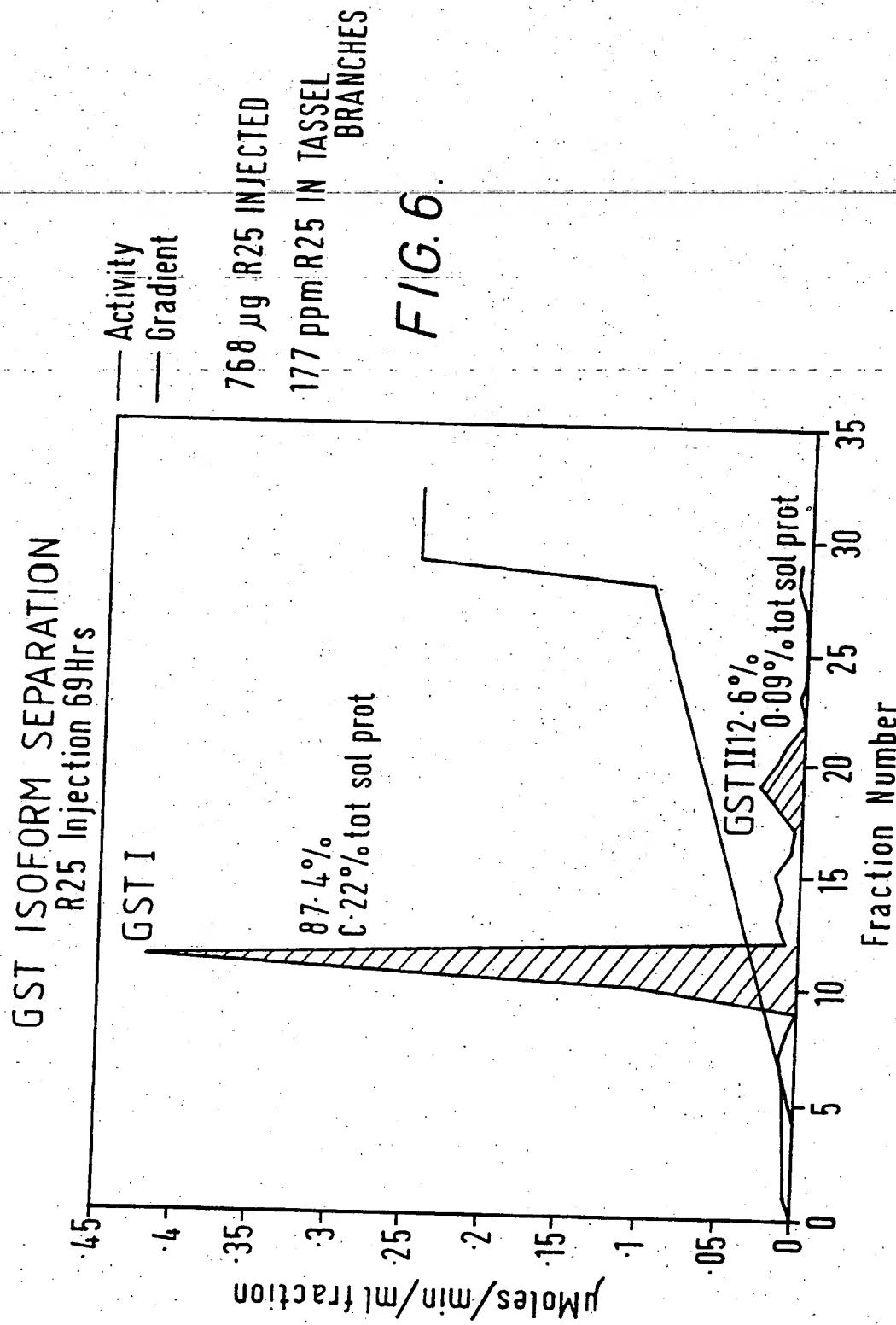
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GST II activity is stimulated in anthers fed with R25 from a stem reservoir

GST ISOFORM SEPARATION

R25 Reservoir (injection) 60 Hrs

—— Activity
—— Gradient

100 ppm R25 IN UPTAKE & MEDIA

8 ppm R25 IN MID FLORETS

FIG. 7.

GST I

84·3%
0·49% of tot sol prot in crude extract

GST II

15·7% 0·26%

Fraction Number

μMoles/min/ml fraction

0   ·2   ·4   ·6   ·8   1   1·2

0   5   10   15   20   25   30   35

SUBSTITUTE SHEET

GST II activity is stimulated in anthers sprayed directly with R25

GST ISOFORM SEPARATION
FLORETS FROM TASSEL SPRAYED WITH R25 72Hrs

FIG. 8.

FIG. 9.

GRAPH TO SHOW THE EFFECT OF INCREASING
INCUBATION TIME WITH R25788 ON TOTAL
GST ACTIVITY

CDNB CONVERTED umoles/min/g fresh weight

INCUBATION TIME WITH R25788 in hours

UNTREATED   TREATED

Approx 2.5 fold increase in total GST activity
at 48 hours.
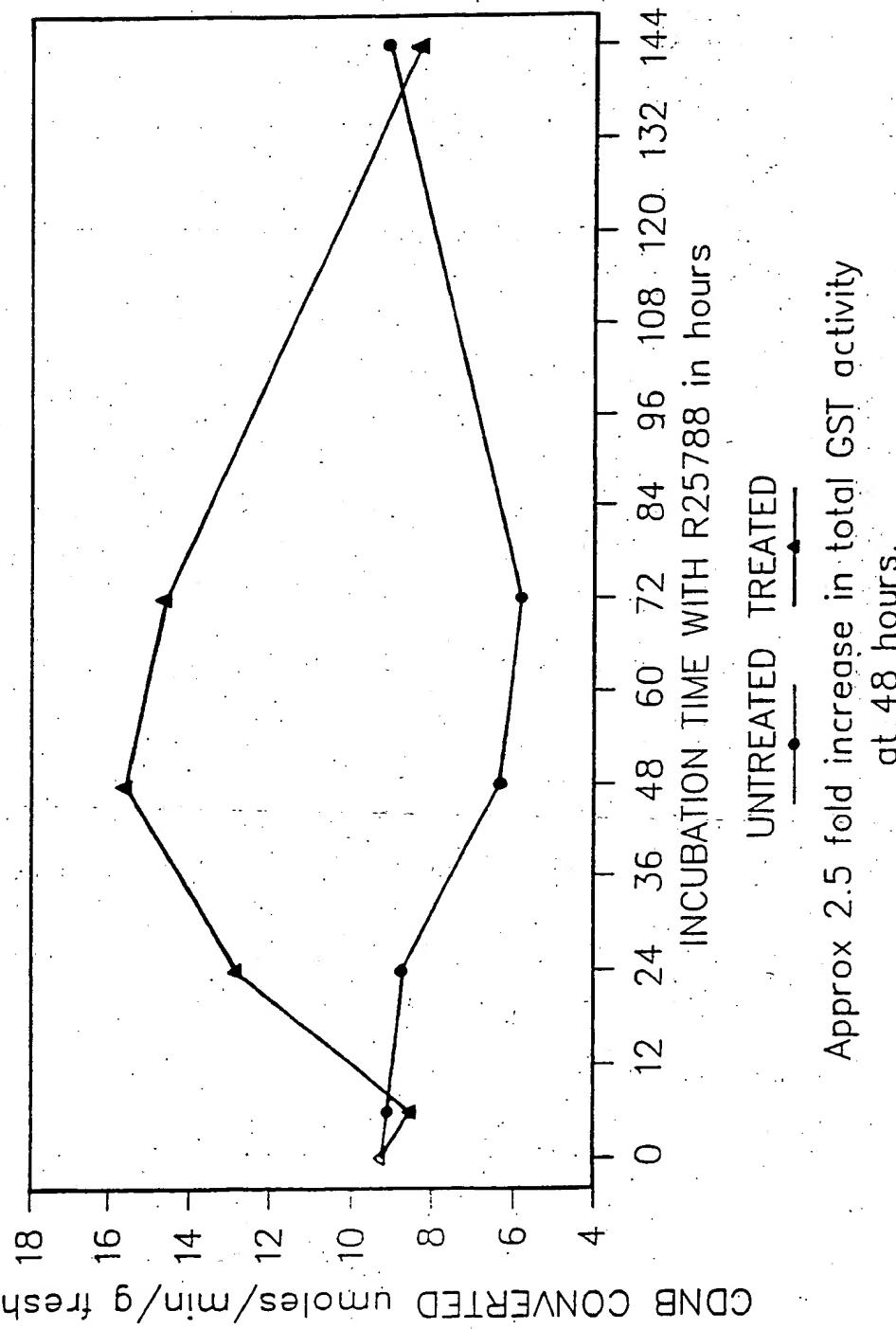
FIG. 10. Cloning PCR-amplified lacI
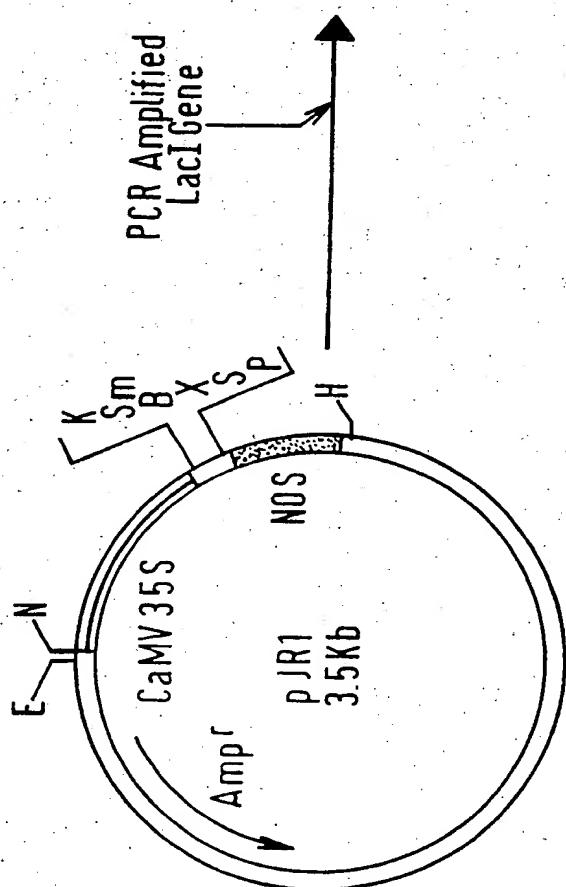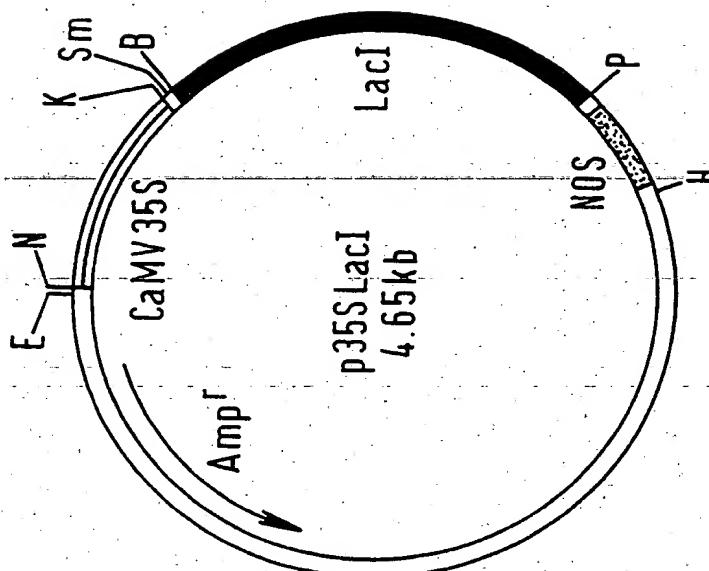
FIG. 11.
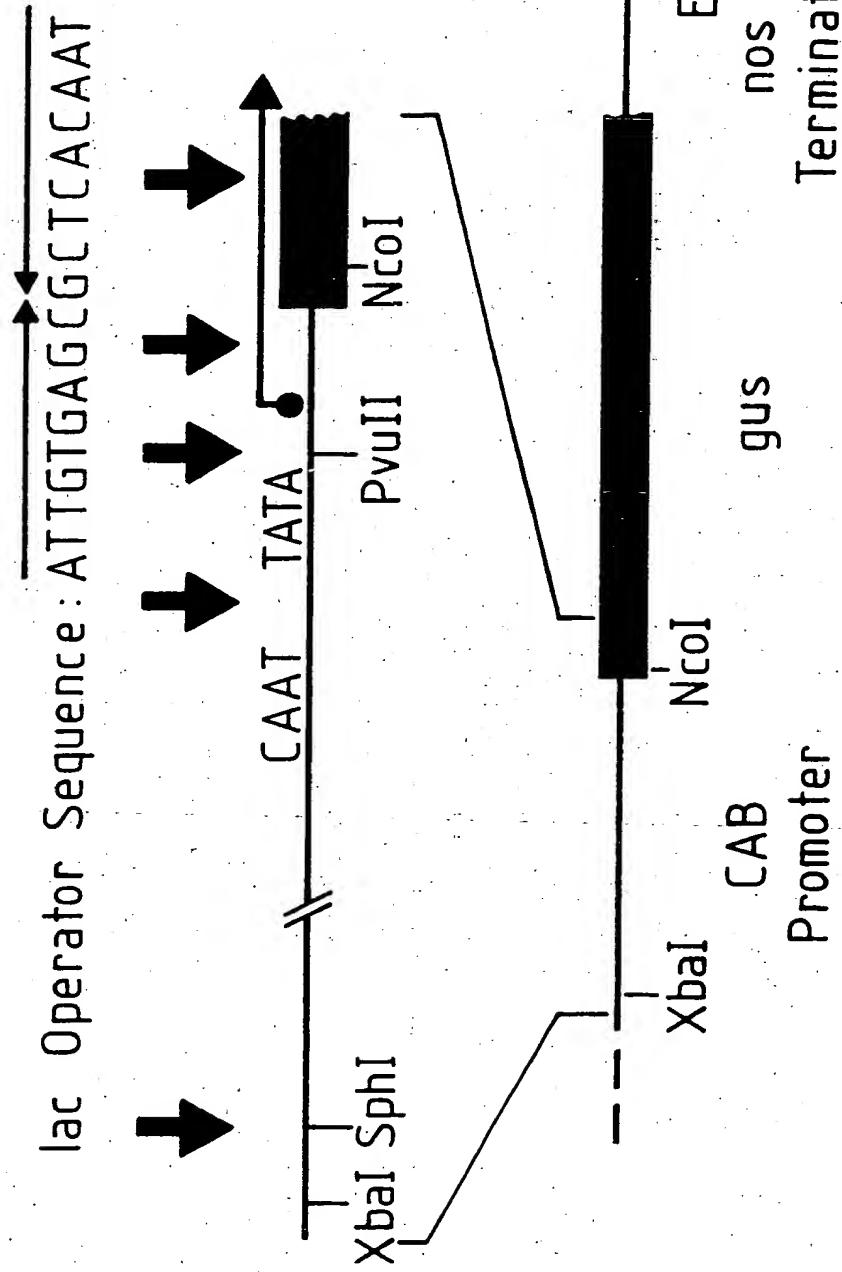
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SEQUENCE OF THE XbaI - NcoI CAB PROMOTER FRAGMENT.

```
              10        20        30        40        50
      XbaI     :         :         :         :         :
   1  GTCTAGATATGTTTCTCTTTTGGTTCTTGAGTTGACAAGTGGCATGCTAT
  51  TTTTGCTCATGTGAGAAAAAATATAGCACCGTTTGTTTCGTTGAAATTGA
 101  ATTGCATCCCAATAATCATAATTTAGACATAAACCAACTAAGTTAATATA
 151  TTTGTATATGTAAATATGTTATCCTAAATTATAATTATATGAGAGAGATA
 201  GTTATACATTACAATTATGATATAGAGAAGCAAATAGAAGAGTGTGCTAT
 251  AAGTTGTACATTGTAAAAGTAGTATGCAAATTTATAGAATTAATTTTTAT
 301  CTTTCACCTCATAAATTTAAGATACACTTATATATAAACTTTGAAAAGTT
 351  GTAAAATATCATATTCTAAAAAAAATAGACTATTCTATTAGTTAGATTCC
 401  AATTCCTTAAAATAAAAGAAAATAAATGGGCTTAAGGGCTAGTTTGGTGG
 451  GCAGGTGGAGGCGATCAATGGCGGATGAATCCTCTCGATATTCAATTGAG
 501  GGGGTTCATCCCATGAATCTCTCTATTTACATGTCCACCAAACAAGATTA
 551  AATGCAATGATACATAGCTTTTGTGCATATTTTTTTTTAACGGAGATGCA
 601  AAACAGTATACAAGATGTGTGTATGTTCCCATGCGACCCATCTTGTATCC
 651  AGTTCATGCGTCTGGTGGTTGAACCAGAAAGATACACATGATCCATTTCG
 701  ATGAATCAACACAAAAGACTTCATTGATTATGCATTTGACAAATAGCAAG
 751  AAGCGCGGGGCAACAAAGCCCATCCTATCTCATCCATTTTTTCCCACGAT
 801  GGCAAGTGGCAGCTCCTGATTAGCTACGCCCATTCCTATGCTATGTGGCA
 851  CACCCCAGGATTCTTGTGTGATAGGCCATTGGGGCCACGAGGAGCCACGT

 901  CAGACGCCAAGCCACCCCGGCGAGAC̲C̲A̲A̲C̲C̲AGC̲C̲A̲A̲TCGCAGTTCAGGA

 951  AAAGATGACCTGCTATCCAAACCCAACTG̲T̲A̲T̲A̲A̲AGGCAGCTGCTGTGT
                    *  * *     *
1001  TCTGTTATGACACAGCCATCACACGCATACTGCATACACAACACAGAGCA
                                  M   A   A   A   T   M
1051  TCAGAAGGAGCTACGATCTGATCGACATGGCTGCCGCCACCATGG
                                               NcoI
```

_____ = CAT and TATA sequences.

   * = Possible transcription start sites.

FIG. 12.

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434 REPRESSOR GENE		SEQUENCE AT *SalI* SITE	
PRESENT	ABSENT		
pPS1	pAD18	GTCGA<u>ACAAGAAAGTTTGT</u>TCGAC	WILD-TYPE 434 O_R1
pPS2	pAD16	GTCGA<u>ACAATATATATTGT</u>TCGAC	"WILD-TYPE" SYNTHETIC
pPS3	pAD17	GTCGA<u>TCAATATATATTGA</u>TCGAC	"MUTANT" SYNTHETIC
pAD15.2	pAD14	G...............................TCGAC	NO OPERATOR
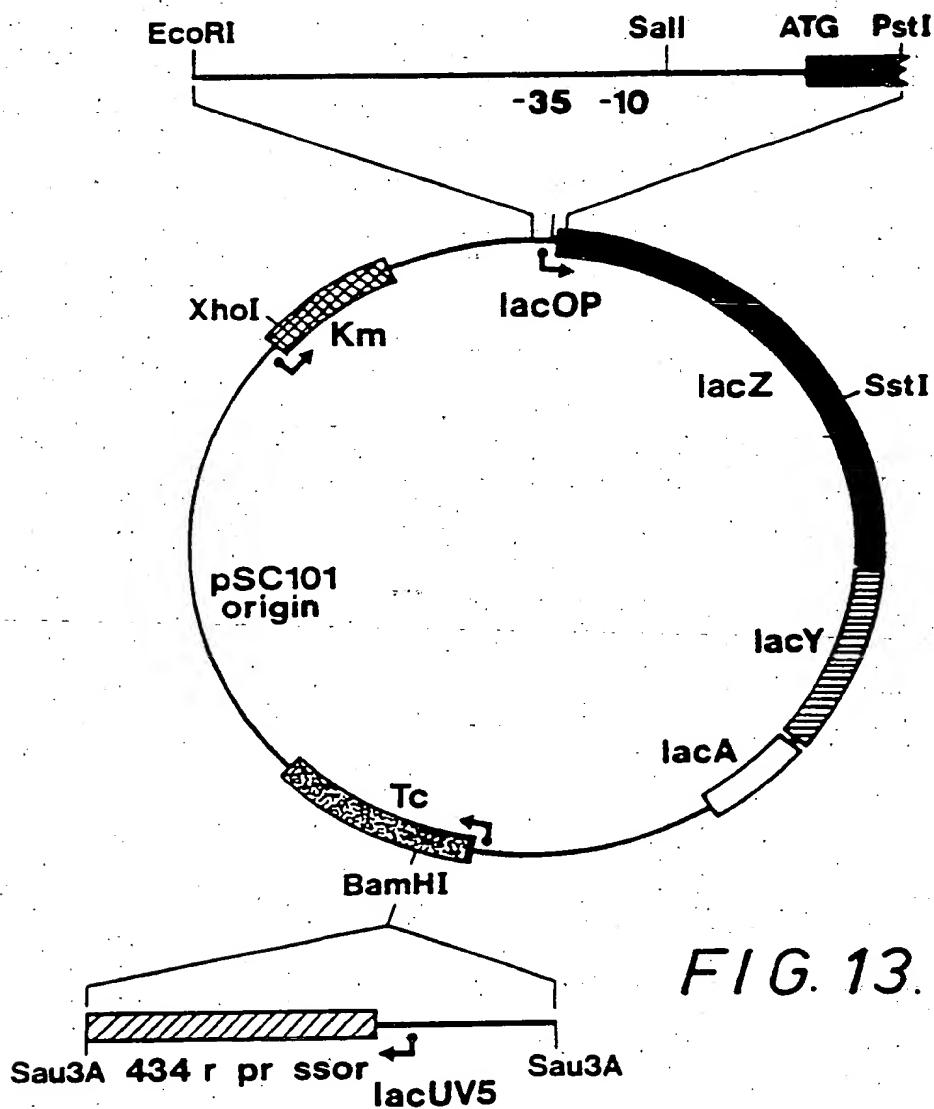
FIG. 13.

ISOLATION OF cDNA CLONES

Whole maize tassels

↓

poly A +mRNA

FIG.14.

cDNA synthesis

↓

10000 pBR322 clones

↓

differential library screening

↓

100 - 200 possible MFS cDNAs

↓

second differential screen

↓

Southern analysis of cDNA
with differential probes

↓

cross hybridisation of clones

↙   ↘

5 early meiotic cDNAs   **1 late meiotic cDNA**
pMS1, pMS2, pMS4   **pMS14**
pMS10, pMS18

FIG. 15.

A
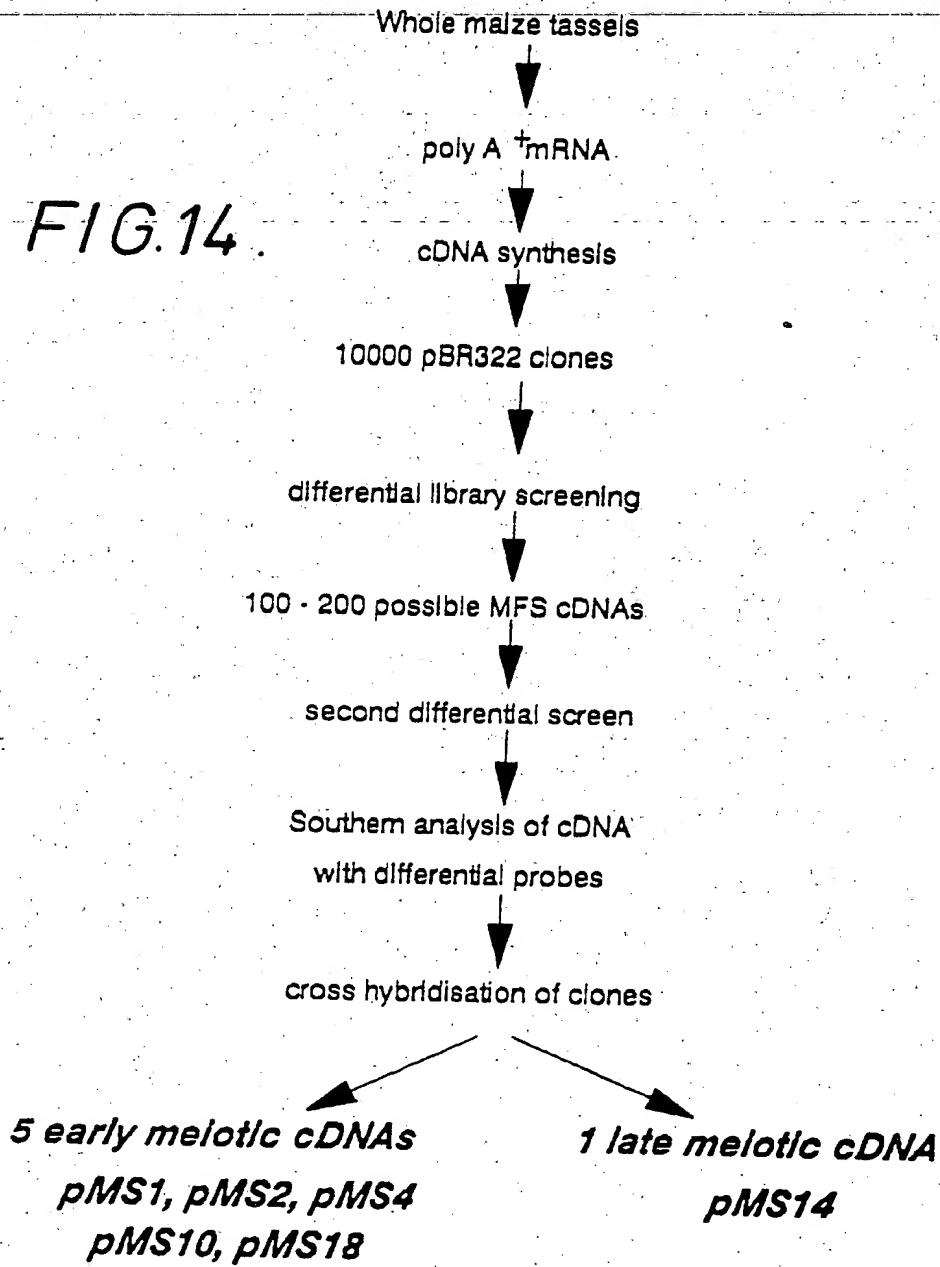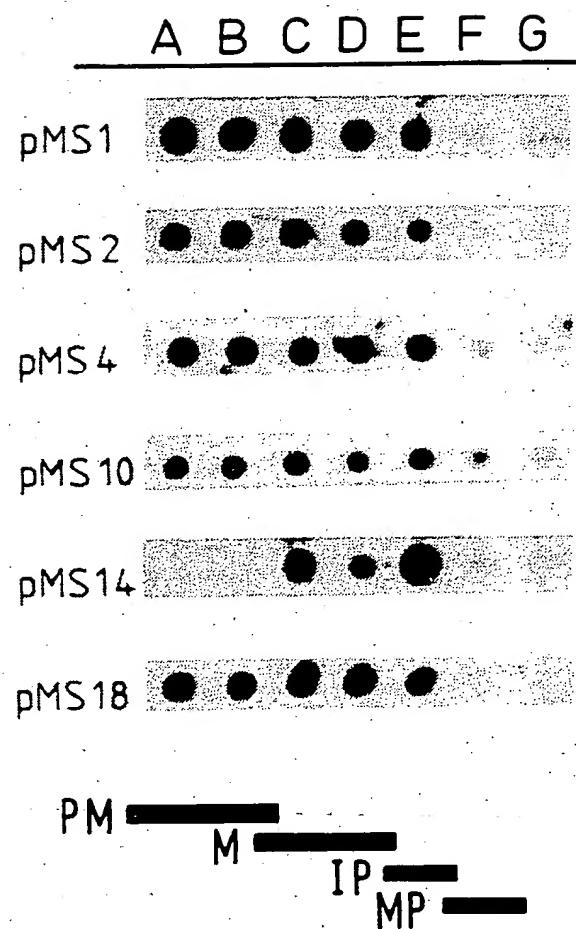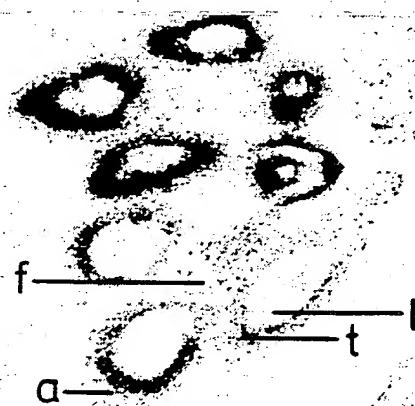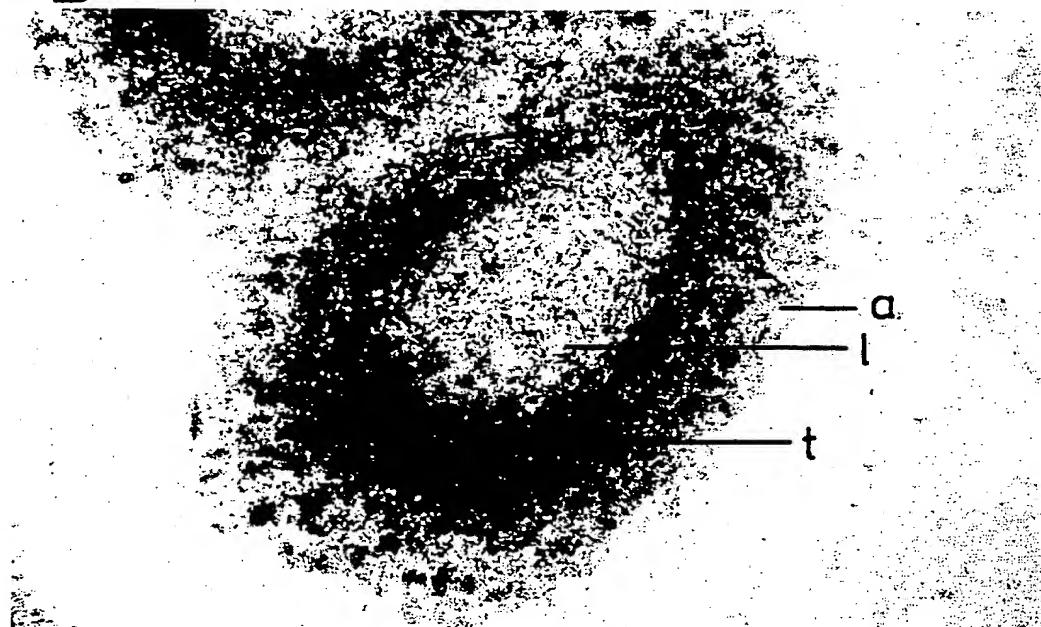
FIG. 16A.

FIG. 16B.

FIG. 16C.

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FIG. 17.

Nucleotide and deduced amino acid sequence for male flower specific cDNA clone, pMS10.

```
         10              20              30              40
         |               |               |               |
GGC CTT GCC CGC TCG TTC CCC TCG CCT CCC CGG TCG CGC CGC TCC

         50              60              70              80              90
         |               |               |               |               |
CGC TGC CGC CGT GGC GAT TCC TGC CCG GCG GCG GCG CCG GGT TCA

            100             110             120             130
            |               |               |               |
GGT CCA CGG CGG CGG CGG CTG CGC GGG GCG GGA CCG ACT ATG GGA
                                                        Met Gly

    140             150             160             170             180
    |               |               |               |               |
CGG ACG ACA GCA AGA TCT CCC CCG ACG AGG AAT TCC TTC GAG GGC
Arg Thr Thr Ala Arg Ser Pro Pro Thr Arg Asn Ser Phe Glu Gly

             190             200             210             220
             |               |               |               |
TGC GAC TAC AAC CAC TGG CTC ATC ACC ATG GAC TTC CCG GAC CCC
Cys Asp Tyr Asn His Trp Leu Ile Thr Met Asp Phe Pro Asp Pro

    230             240             250             260             270
    |               |               |               |               |
AAG CCG TCG CGC GAA GAG ATG ATC GAG ACA TAC CTC CAG ACT CTC
Lys Pro Ser Arg Glu Glu Met Ile Glu Thr Tyr Leu Gln Thr Leu

             280             290             300             310
             |               |               |               |
GCC AAG GTC GTC GGG AGT TAT GAG GAG GCC AAG AAG AGG ATG TAT
Ala Lys Val Val Gly Ser Tyr Glu Glu Ala Lys Lys Arg Met Tyr

    320             330             340             350             360
    |               |               |               |               |
GCT TTT AGT ACG ACG ACT TAT GTT GGT TTT CAG GCT GTA ATG ACC
Ala Phe Ser Thr Thr Thr Tyr Val Gly Phe Gln Ala Val Met Thr

             370             380             390             400
             |               |               |               |
GAG GAA ATG TCA GAA AAA TTT CGC GGT TTG CCT GGA GTA GTT TTC
Glu Glu Met Ser Glu Lys Phe Arg Gly Leu Pro Gly Val Val Phe

    410             420             430             440             450
    |               |               |               |               |
ATT TTG CCT GAT TCA TAT CTA TAT CCA GAA ACA AAG GAG TAC GGA
Ile Leu Pro Asp Ser Tyr Leu Tyr Pro Glu Thr Lys Glu Tyr Gly

             460             470             480             490
             |               |               |               |
GGA GAC AAA TAT GAC AAT GGT GTC ATC ACT CCA AGA CCA CCA CCT
Gly Asp Lys Tyr Asp Asn Gly Val Ile Thr Pro Arg Pro Pro Pro
```

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FIG. 17.
(cont.)

```
        500              510               520              530              540
         |                |                 |                |                |
GTT CAT TAT AGC AGA CCA TCA AGA ACT GAC AGG AAC CGT AAC TAC
Val His Tyr Ser Arg Pro Ser Arg Thr Asp Arg Asn Arg Asn Tyr

                 550              560              570              580
                  |                |                |                |
CGA GGA AAC TAC CAG GAT GGC CCT CCA CAG CAA GGA AAT TAC CAG
Arg Gly Asn Tyr Gln Asp Gly Pro Pro Gln Gln Gly Asn Tyr Gln

        590              600              610              620              630
         |                |                |                |                |
AAC AAC CGT CCT CCA CCA GAA GGT GGT TAC CAG AAC AAC CCG CCG
Asn Asn Arg Pro Pro Pro Glu Gly Gly Tyr Gln Asn Asn Pro Pro

                 640              650              660              670
                  |                |                |                |
CAG CAA GGA AAC TAC CAG ACA TAC CGC TCG CAG CAA GAT GGA AGA
Gln Gln Gly Asn Tyr Gln Thr Tyr Arg Ser Gln Gln Asp Gly Arg

        680              690              700              710              720
         |                |                |                |                |
GGC TAT GCC CCA CAG CAG AAT TAT GCA CAA GGT GGT CAG GAT GGT
Gly Tyr Ala Pro Gln Gln Asn Tyr Ala Gln Gly Gly Gln Asp Gly

                 730              740              750              760
                  |                |                |                |
AGA GGT TTT GGA AGG AAT GAT TAC ACA GAC CGT TCA GGC TAC AAT
Arg Gly Phe Gly Arg Asn Asp Tyr Thr Asp Arg Ser Gly Tyr Asn

        770              780              790              800              810
         |                |                |                |                |
GGA CCC ACT GAT TTT CGA AGT CAA ACT CAG TAC CAA GGG CAT GTA
Gly Pro Thr Asp Phe Arg Ser Gln Thr Gln Tyr Gln Gly His Val

                 820              830              840              850
                  |                |                |                |
AAT CCA GCT GGG CAA GGT CAA GGT TAC AAC AAC CCC CAA GAG CGT
Asn Pro Ala Gly Gln Gly Gln Gly Tyr Asn Asn Pro Gln Glu Arg

        860              870              880              890              900
         |                |                |                |                |
ACG AAC TTC TCG CAA GGG CAG GGA GGA GGT TTT AGG CCT GGT GGT
Thr Asn Phe Ser Gln Gly Gln Gly Gly Gly Phe Arg Pro Gly Gly

                 910              920              930              940
                  |                |                |                |
CCT TCA GCA CCT GGG TCT TAT GGC CAA CCA TCA GCA CCT GGA TCT
Pro Ser Ala Pro Gly Ser Tyr Gly Gln Pro Ser Ala Pro Gly Ser

        950              960              970              980              990
         |                |                |                |                |
TAT GGT CAA CCT AAT ACA CTT GGT AAC TAT GGG CAG GTA CCT CCA
Tyr Gly Gln Pro Asn Thr Leu Gly Asn Tyr Gly Gln Val Pro Pro
```

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FIG. 17.
(cont.)

```
        1000              1010            1020              1030
         |                 |               |                 |
TCA GTG AAT CCT GGT GGT AAC AGA GTT CCT GGT GTG AAT CCT AGT
Ser Val Asn Pro Gly Gly Asn Arg Val Pro Gly Val Asn Pro Ser

    1040            1050            1060            1070            1080
     |               |               |               |               |
TAT GGT GGG GAT GGC AGA CAG GGG GCT GGA CCA GCA TAT GGT GGA
Tyr Gly Gly Asp Gly Arg Gln Gly Ala Gly Pro Ala Tyr Gly Gly

        1090            1100            1110            1120
         |               |               |               |
GAT AAC TGG CAA AGA GGT TCT GGT CAG TAT CCT AGC CCA GGT GAA
Asp Asn Trp Gln Arg Gly Ser Gly Gln Tyr Pro Ser Pro Gly Glu

    1130            1140            1150            1160            1170
     |               |               |               |               |
GGA CAA GGA AAC TGG CAG GGA AGG CAG TAA GAG CTG ACG TGT TCC
Gly Gln Gly Asn Trp Gln Gly Arg Gln

        1180            1190            1200            1210
         |               |               |               |
ACT GAA GAC AAG AAT GGC ACT TGA GAT TTA GAA ATC TCC ATC TGT

    1220            1230            1240            1250            1260
     |               |               |               |               |
AAA ATA AAC GAC TGT GAT GCA TTA CTC TTT TTT TTT TTC TTG CAT

        1270            1280            1290            1300
         |               |               |               |
TTG AAC TCT AAA CTT ATG GGC ATG CGT TAT TAC CAA ACT ACG GAT

    1310            1320            1330            1340            1350
     |               |               |               |               |
GCA AAT TCA TTT TAG TTT TTT GGG CCA AAT GTT GGC ATT TTT AAA

AAA
```

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FIG. 18.

Nucleotide and deduced amino acid sequence for the
male flower specific cDNA clone, pMS14.

```
              10             20             30             40
              |              |              |              |
GCA GGG GGG GGG GCA CAG CAA GCC AGC AGA GCA GAA AGC AGC CGC
Ala Gly Gly Gly Ala Gln Gln Ala Ser Arg Ala Glu Ser Ser Arg

         50             60             70             80             90
         |              |              |              |              |
AGC CCC AGC CCC CAC AAA GAC GAA GGC AAC AAT GGC GCT AGA AGC
Ser Pro Ser Pro His Lys Asp Glu Gly Asn Asn Gly Ala Arg Ser

              100            110            120            130
              |              |              |              |
AGC CAC GCC CCC CGC GCA CTC CTC GCG CGT GCC TCG TCC TGC TGG
Ser His Ala Pro Arg Ala Leu Leu Ala Arg Ala Ser Ser Cys Trp

     140            150            160            170            180
     |              |              |              |              |
TCC TCG GCG GCG GCA CCG GCC CGT CGT CGG TGC TCA GCG CGC CGG
Ser Ser Ala Ala Ala Pro Ala Arg Arg Arg Cys Ser Ala Arg Arg

              190            200            210            220
              |              |              |              |
GGC GCA GGA CCG GCG GCA GTG CCT GCC GCA GCT GAA CGC CTC CTG
Gly Ala Gly Pro Ala Ala Val Pro Ala Ala Ala Glu Arg Leu Leu

     230            240            250            260            270
     |              |              |              |              |
CGG TGC CGC GCG TAC CTG GTG CCG GCG CGC CGG ACC CCA GCG CGG
Arg Cys Arg Ala Tyr Leu Val Pro Ala Arg Arg Thr Pro Ala Arg

              280            290            300            310
              |              |              |              |
ACT GCT GCA GCG CTG ACG CGC CGT GTG CAC GAG TGC GCC TGC AGC
Thr Ala Ala Ala Leu Thr Arg Arg Val His Glu Cys Ala Cys Ser

     320            330            340            350            360
     |              |              |              |              |
ACC ATG GGC ATC ATC AAC AGC CTG CCC GGC CGG TGC CAC CTC GCC
Thr Met Gly Ile Ile Asn Ser Leu Pro Gly Arg Cys His Leu Ala

              370            380            390            400
              |              |              |              |
CAA GCC AAC TGC TCC GCT TGA AGC AGG GAC CTG GCA CGC GTG CTG
Gln Ala Asn Cys Ser Ala

     410            420            430            440            450
     |              |              |              |              |
CAA TGG ATG GCA GGA GGG GAG AGG AAT AAG AAG TGT TTC CAT TTC

              460            470            480            490
              |              |              |              |
ACA GTG AGA GCA GTC GAG CTC CAA CGT TGT CGT CGT CGT CGT CTT
```

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FIG. 18.
(cont.)

```
      500              510              520              530              540
       |                |                |                |                |
CTT CTT TTG ATA TTC AGA CTC TGT CTT GCG GTC TAT ATC ATC AGC
          550              560              570              580
           |                |                |                |
ATA ATA ATA ATA AAA TAA GTA AAA CCA AAA AAA AAA AAA AA
```

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FIG.19.

Nucleotide and deduced amino acid sequence for the
male flower specific cDNA clone, pMS18.

```
              10          20          30          40
               |           |           |           |
ACA GCA GTA GCA AGA GGG ATA GAG CAA GGC CAC ACA CAC ACA CAC

        50          60          70          80          90
         |           |           |           |           |
ACC ACT AGG CTA GGT TAG CCT TTT AAT CGT CGT CGA GAA GCA AGA

            100         110         120         130
             |           |           |           |
AGG GCG CTG CAC CAA GCA GGC AAG CAA GAA GAG AGC CGA TCG ACC

     140        150         160         170         180
      |          |           |           |           |
GAG AGC TAG CAC GCG ATG GCG AGG TCT TGC CAA GAT GAT GGT GGC
                    Met Ala Arg Ser Cys Gln Asp Asp Gly Gly

            190         200         210         220
             |           |           |           |
GCA CGT CTG CTG GCC TTG CGC TGG CGT GTC GAC CGC CGA GGC AGG
Ala Arg Leu Leu Ala Leu Arg Trp Arg Val Asp Arg Arg Gly Arg

    230        240         250         260         270
     |          |           |           |           |
AAC ATC AAG ACC ACG ACG ACG GAG AAG AAG GAC GAC GCG GTG GTG
Asn Ile Lys Thr Thr Thr Thr Glu Lys Lys Asp Asp Ala Val Val

            280         290         300         310
             |           |           |           |
CAG CCG CAG AGG TTC CGC CCT TCG ACC GCC TCG GCG CGG CGC GTC
Gln Pro Gln Arg Phe Arg Pro Ser Thr Ala Ser Ala Arg Arg Val

    320        330         340         350         360
     |          |           |           |           |
CCC GGC GTT CGG CGG CCT CCC CGG CGG CAC GAT TCC TGG CAG CAG
Pro Gly Val Arg Arg Pro Pro Arg Arg His Asp Ser Trp Gln Gln

            370         380         390         400
             |           |           |           |
CAT TCC CGG GTT CAG CAT GCC CGG CAG CGG CAG CAG CCT ACC CGG
His Ser Arg Val Gln His Ala Arg Gln Arg Gln Gln Pro Thr Arg

    410        420         430         440         450
     |          |           |           |           |
GTT CAG CTT GCC CGG CAG CGG CAC GAT GCC CCT CTT CGG CGG CGG
Val Gln Leu Ala Arg Gln Arg His Asp Ala Pro Leu Arg Arg Arg

            460         470         480         490
             |           |           |           |
CTC CCC GGG CTT CAG CGG CTT CGG CGG CAT GCC CGG GTC GCC CAC
Leu Pro Gly Leu Gln Arg Leu Arg Arg His Ala Arg Val Ala His
```

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FIG.19.

(cont.)

```
          500          510          520          530          540
           |            |            |            |            |
CGC CGG CTC CGT CCC CGA GCA CGC CAA CAA GCC CTG AAC GCC AAC
Arg Arg Leu Arg Pro Arg Ala Arg Gln Gln Ala Leu Asn Ala Asn

               550          560          570          580
                |            |            |            |
AAG CGT GGT AGT AGA GGT GCT ACT GTT ACT GTA GTA CGT CGT CGT
Lys Arg Gly Ser Arg Gly Ala Thr Val Thr Val Val Arg Arg Arg

     590          600          610          620          630
      |            |            |            |            |
CTT CAT GCA TGC GTG GTT CGT GGT TTC CCT AGC TCC ATA CGA GCA
Leu His Ala Cys Val Val Arg Gly Phe Pro Ser Ser Ile Arg Ala

               640          650          660          670
                |            |            |            |
GTA GTT GGG CTT GCA CGT ACC GTA CGT CTA GCT AGC TAT ATA TAT
Val Val Gly Leu Ala Arg Thr Val Arg Leu Ala Ser Tyr Ile Tyr

     680          690          700          710          720
      |            |            |            |            |
GCT TGT GTT CTA CTG CTT TTT AGT TTA ATT ACC TGC CTG CAT TGG
Ala Cys Val Leu Leu Leu Phe Ser Leu Ile Thr Cys Leu His Trp

               730          740          750          760
                |            |            |            |
AGA GTT GGA TCT GTT TCA TTT GGT GGT GTT TGC TTT ACT ATT AGG
Arg Val Gly Ser Val Ser Phe Gly Gly Val Cys Phe Thr Ile Arg

     770          780          790          800          810
      |            |            |            |            |
TCA GTA TCT GTT TGT GGA GAC TTG GTG TTT AAT TTA TTT AGC CGT
Ser Val Ser Val Cys Gly Asp Leu Val Phe Asn Leu Phe Ser Arg

               820          830          840          850
                |            |            |            |
TTG TGA CTG GTT GTA GCT AGC GGT GGT GCG GTG GTG ATG TTC TTG
Leu

     860          870          880          890          900
      |            |            |            |            |
AGG CAT GAA TAA TGC TAC ATG CAT GTG ATG TAT CCA TGT TTT GTG

               910          920          930
                |            |            |
TGT GGT AAA CCT GTT GTT TGT ATA AGC TGT CCC
```

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Restriction map of 9 kb EcoRI fragment from clone 10/CT8-3
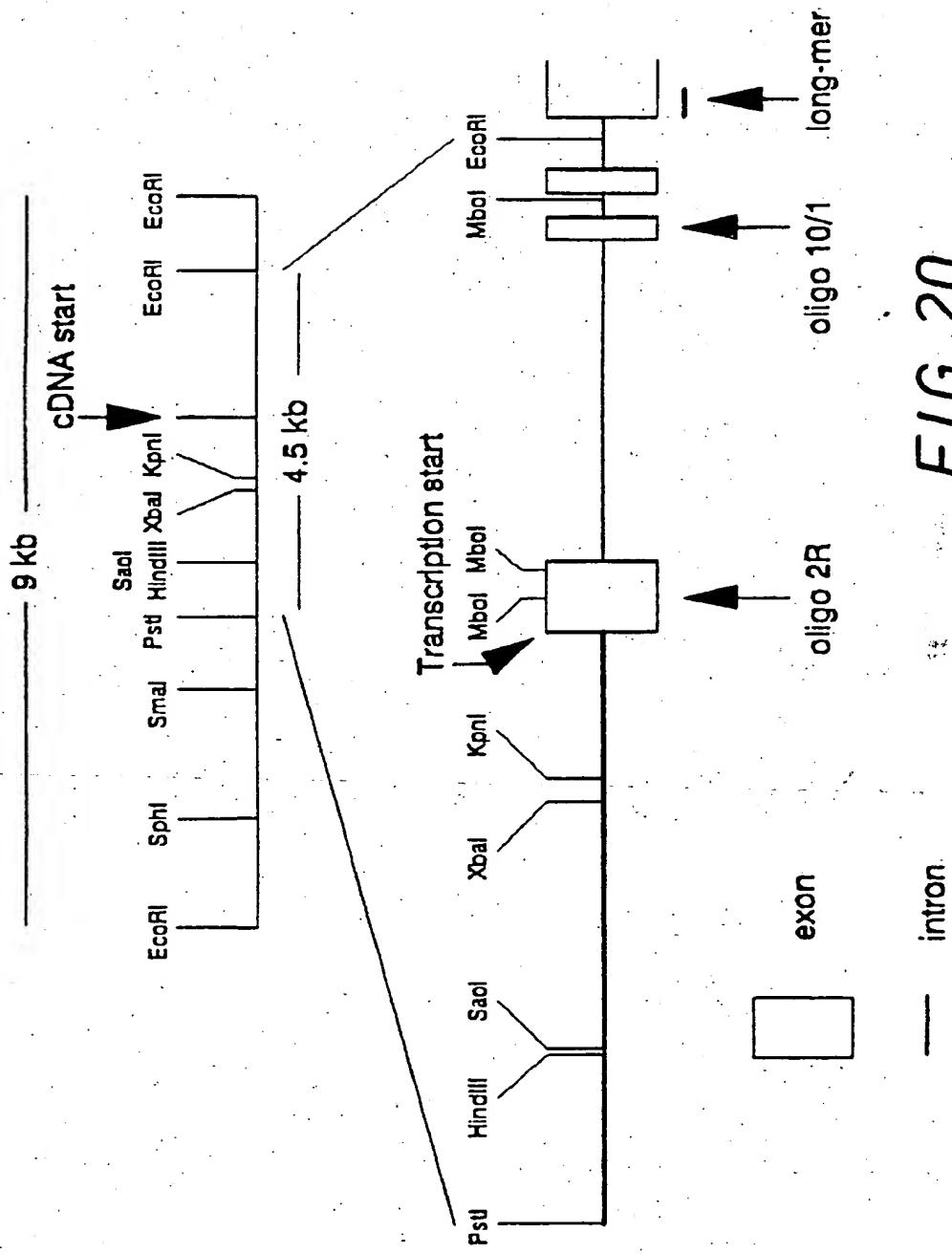
FIG. 20.

Restriction map of 17 kb EcoRI fragment from clone 14/17M
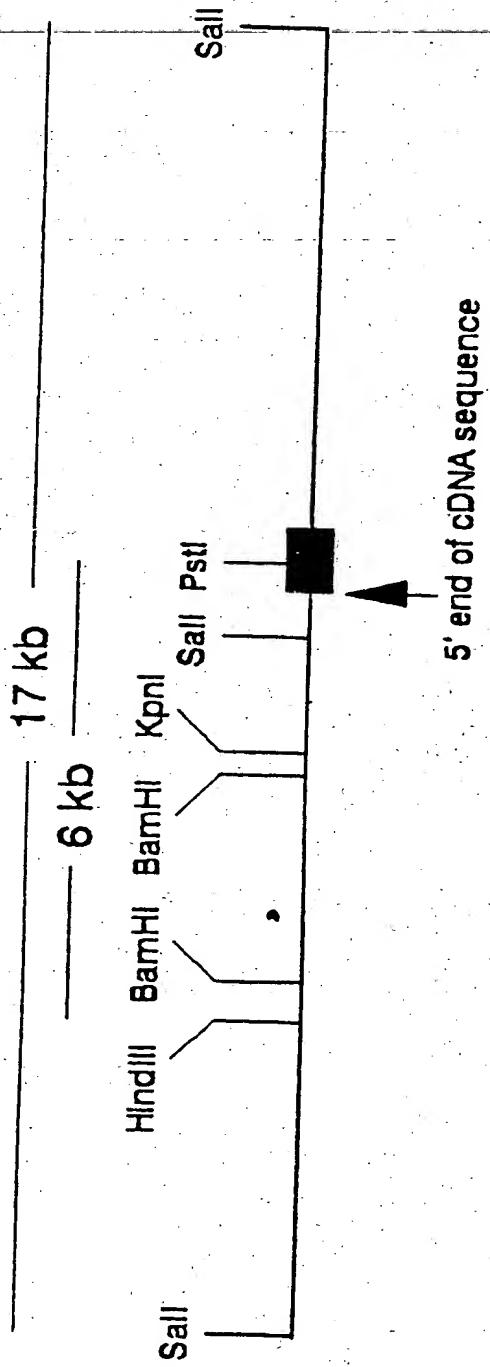
FIG. 21.

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Restriction map of 16 kb EcoRI fragment from clone 18/CT3

region corresponding to pMS18

FIG. 22.

16 kb

Sal I   Sma I   BamHI   Sma I   Sal I   BamHI   BamHI   Sal I

BamHI   SphI   EcoRI   HindIII   SphI   Sal I

3.5 kb

subcloned into pUC19
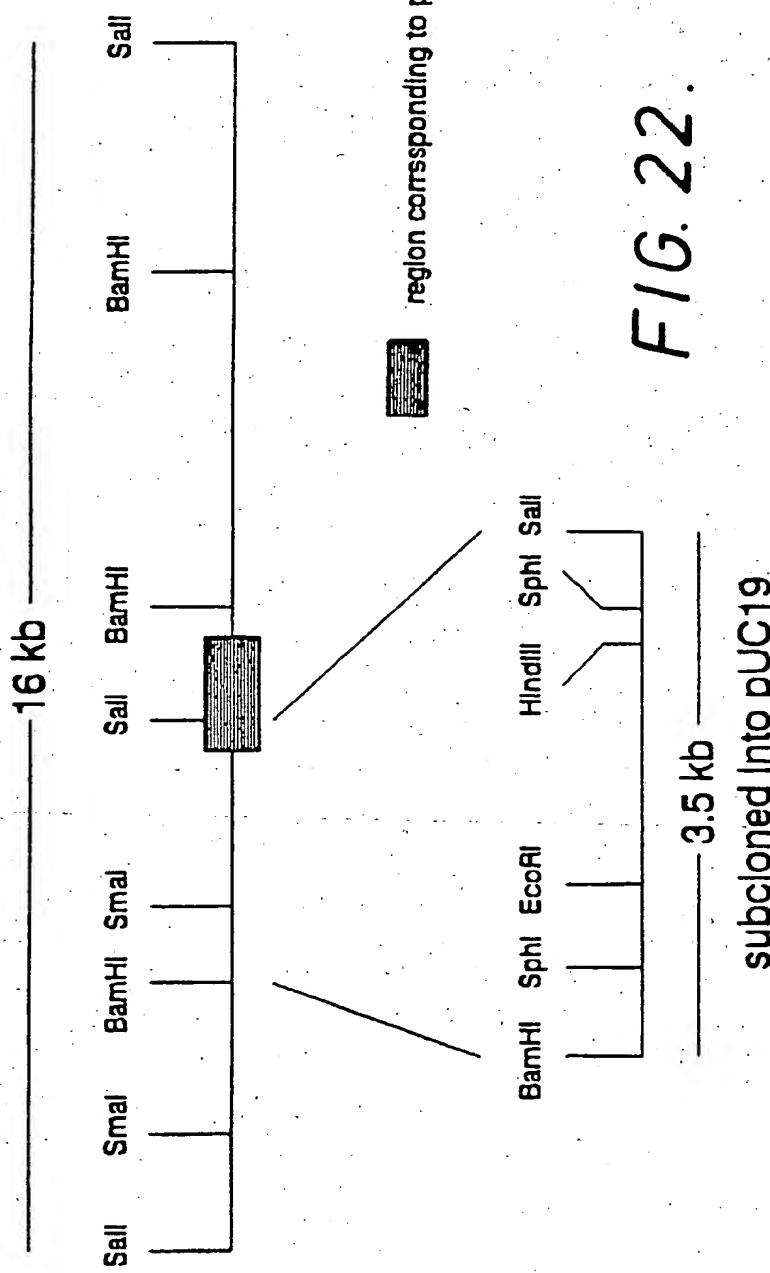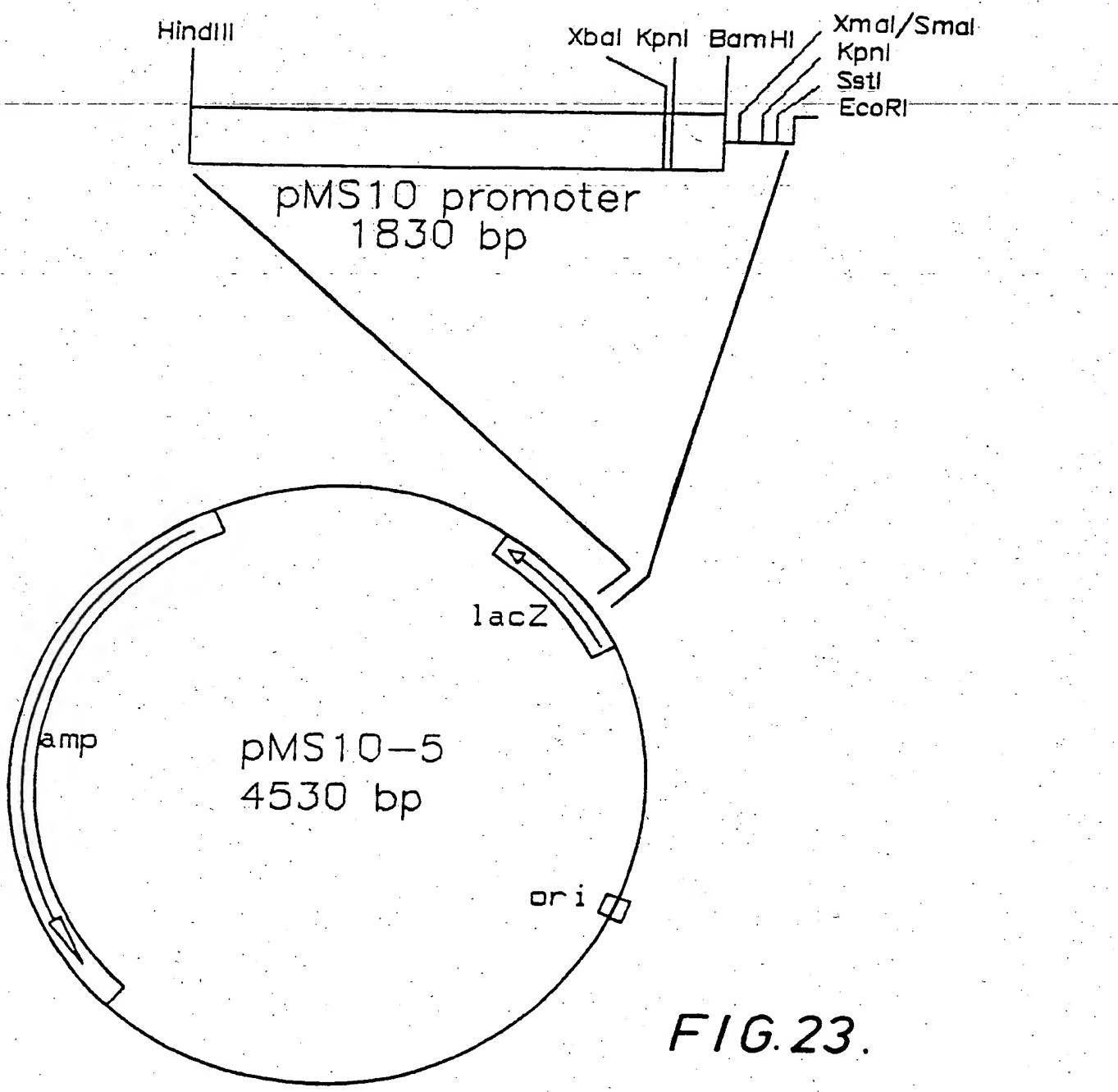
FIG.23.

Clone pMS10-5.

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FIG. 24.

Structure of pTAK1, pTAK2, pTAK3

SstI   EcoRI

Bin19 ←   1800bp |250 bp| → Bin19

β-glucuronidase   nos-terminator

SphI   SalI   BamHI

HindIII   PstI   XbaI   SmaI

Hind III - SmaI linker is from pUCI9

Hind III, Sal I, Xba I, BamHI, SmaI are unique in the vector
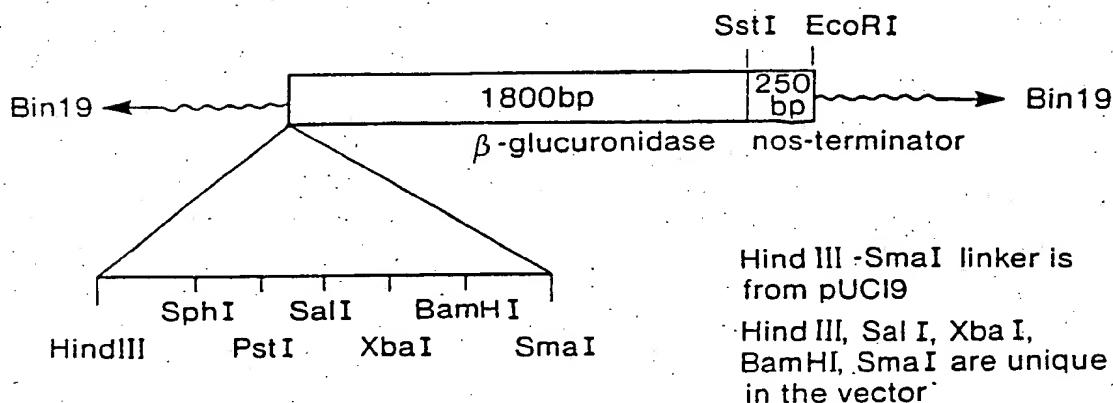
pTAK1 G G A T C C C C G   G G T G G T C A G T C C C T T | ATG |
         BamHI   SmaI

pTAK2 G G A T C C C C G G   G T A G G T C A G T C C C T T | ATG |
                        SmaI

pTAK3 G G A T C C C C G G G   T A C G G T C A G T C C C T T | ATG |
                      SmaI

SUBSTITUTE SHEET

Map of clone pMS10-6GUS
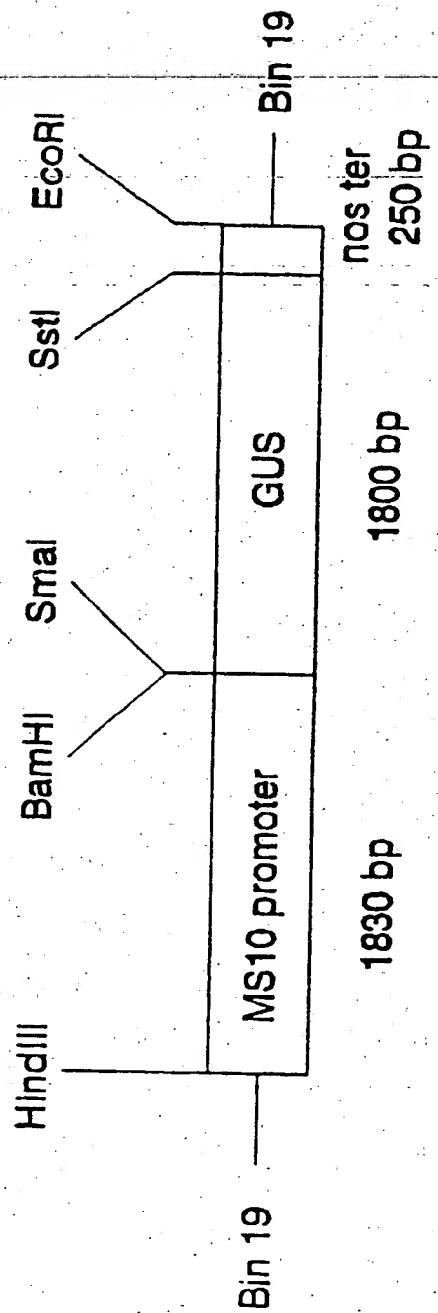
FIG. 25.

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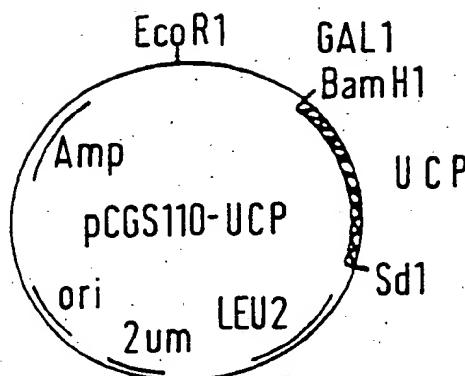
FIG. 26.

FIG. 27

```
              KpnI
   1  UCGAAGUUGA GAGUUCGGUA CCCACAUCAG GCAACAGUGC CACUGUUGUC
  51  UUCAGGGCUG AUUCCUUUUG GUCUCUGCCC UCCGAGCCAA GAUGGUGAGU
 101  UCGACAACUU CCGAAGUGCA ACCCACCAUG GGGGUCAAGA UCUUCUCAGC
 151  CGGCGUUUCU GCCUGCCUAG CAGACAUCAU CACCUUCCCG CUGGACACCG
 201  CCAAAGUCCG CCUUCAGAUC CAAGGUGAAG GCCAGGCUUC CAGUACUAUU
 251  AGGUAUAAAG GUGUCUUAGG GACCAUCACC ACCCUGGCCA AGACAGAAGG
 301  AUUGCCGAAA CUGUACAGCG GUCUGCCUGC UGGCAUCCAG AGGCAAAUCA
 351  GCUUUGCUUC CCUCAGGAUU GGCCUCUACG AUACGGUCCA AGAGUACUUC
 401  UCUUCAGGGA GAGAAACGCC UGCCUCUUUG GGAAGCAAGA UCUCGGCUGG
 451  CUUGAUGACG GGUGGCGUGG CGGUAUUCAU UGGGCAGCCC ACAGAGGUGG
 501  UGAAGGUCAG AAUGCAAGCA CAAAGCCAUC UGCACGGGAU CAAACCCCGC
 551  UACACUGGGA CCUACAAUGC UUACAGAGUU AUAGCCACCA CAGAAAGCUU
 601  GUCAACACUG UGGAAAGGGA CGACUCCUAA UCUAAUGAGA AAUGUCAUCA
 651  UCAACUGUAC AGAGCUGGUG ACAUAUGACC UCAUGAAGGG GGCCCUUGUG
 701  AACCACCACA UACUGGCAGA UGACGUCCCC UGCCAUUUAC UGUCAGCUCU
 751  UGUCGCCGGG UUUUGCACCA CACUCCUGGC CUCUCCGGUG GAUGUGGUAA
 801  AAACGAGAUU CAUCAACUCU CUACCAGGAC AGUACCCAAG UGUACCCAGC
 851  UGUGCAAUGA CCAUGUACAC CAAGGAAGGA CCGGCAGCCU UUUUCAAAGG
 901  GUUUGCGCCU UCUUUUCUGC GACUCGGAUC CUGGAACGUC AUCAUGUUUG
 951  UGUGCUUUGA ACAGCUGAAG AAAGAGCUGA UGAAGUCCCG GCAGACAGUG
1001  GACUGCACCA CAUAGGCGAC UUGGAGAAAG GGAUGCUAAA CACCAUUGGG
1051  CUCCUAUGCU GGGCUCCUAU GCUGGGAGAC CACGAAUAAA ACCAACCAAA
1101  GAAAUCAGAC G
```

YM147 (a) X SF747 (α)

↓

gal⁺, his⁺, trp⁺, leu⁺, ura⁻ (ura selection)

↓

diploids sporulated

↓

Tetrads dissected and mutations of amino acid biosynthesis
determined using replica plating on various selection plates

↓

Strains with trp⁻, leu⁻, ura⁻, gal⁻, phenotype crossed with
strains KY117 and KY118 to determine which gal mutation was present

↓

Selection on galactose medium

BET9 (a)   BE 27 (α)

his4, ura3, trp1, leu2, gal1   ura3, trp1, leu, gal1

FIG.28. Generation of a leu2, gal1 yeast strain.

	BE27	BE27 PKV	BE27 UCP.	BET9	BET9 PKV	BET9 UCP
YPD YPD+Gal	*** ***	*** ***	*** ***	*** ***	*** ***	*** ***
YPDG YPDG+Gal	*** ***	*** ***	*** ***	*** ***	*** ***	*** ***
MMGlu MMGlu+Gal	*** ***	*** ***	*** ***	*** ***	*** ***	*** ***
MMLac MMLac+Gal	*** ***	*** **	*** ***	*** ***	** ***	*** **
MMGly MMGly+Gal	*** ***	*** **	*** **	*** **	*** ***	*** **
LacCas LacCas+Gal	*** ***	*** **	*** ***	*** ***	*** ***	*** ***
GlyCas GlyCasGal	*** ***	*** **	*** *	*** ***	*** ***	*** *
2%Lac 2%Lac+Gal	*** ***	*** ***	*** ***	*** ***	*** ***	*** ***

FIG.29.

FIG. 30.

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FIG. 31.

Time (hours)

FIG. 32.

Time (hours)

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FIG. 33.

FIG.34.

A.

B.

Oligo B1   w.t. ACGGTCAAATGAACGAACCTC
            mut. ACGGTCAAATAAACGAACCTC
                            x

Oligo B2   w.t. AGGTGTCGGTAAGACTGTGTT
            mut. AGGTGTCGGTCAGACTGTGTT
                            x
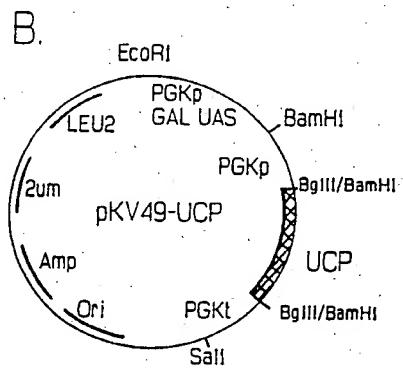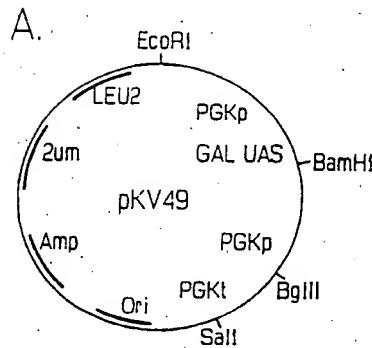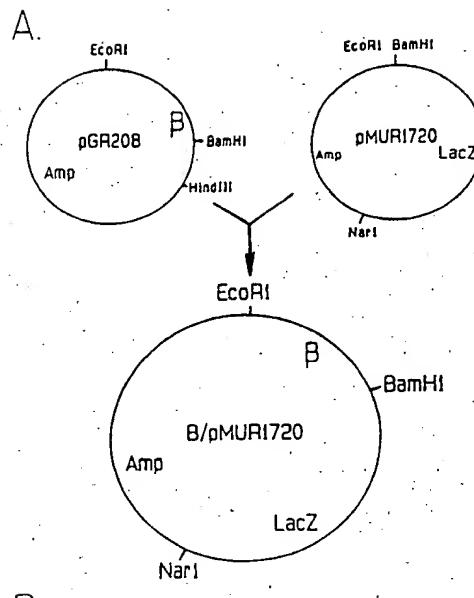
FIG. 35.

A.   B.

FIG. 36. Construction of β-subunit / β-galactosidase fusion protein
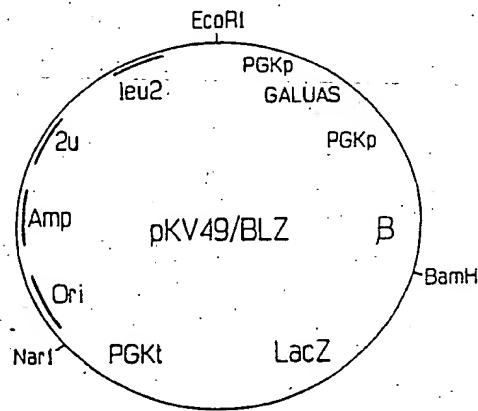
FIG. 37. Plasmid map of the construct pKV49/BLZ
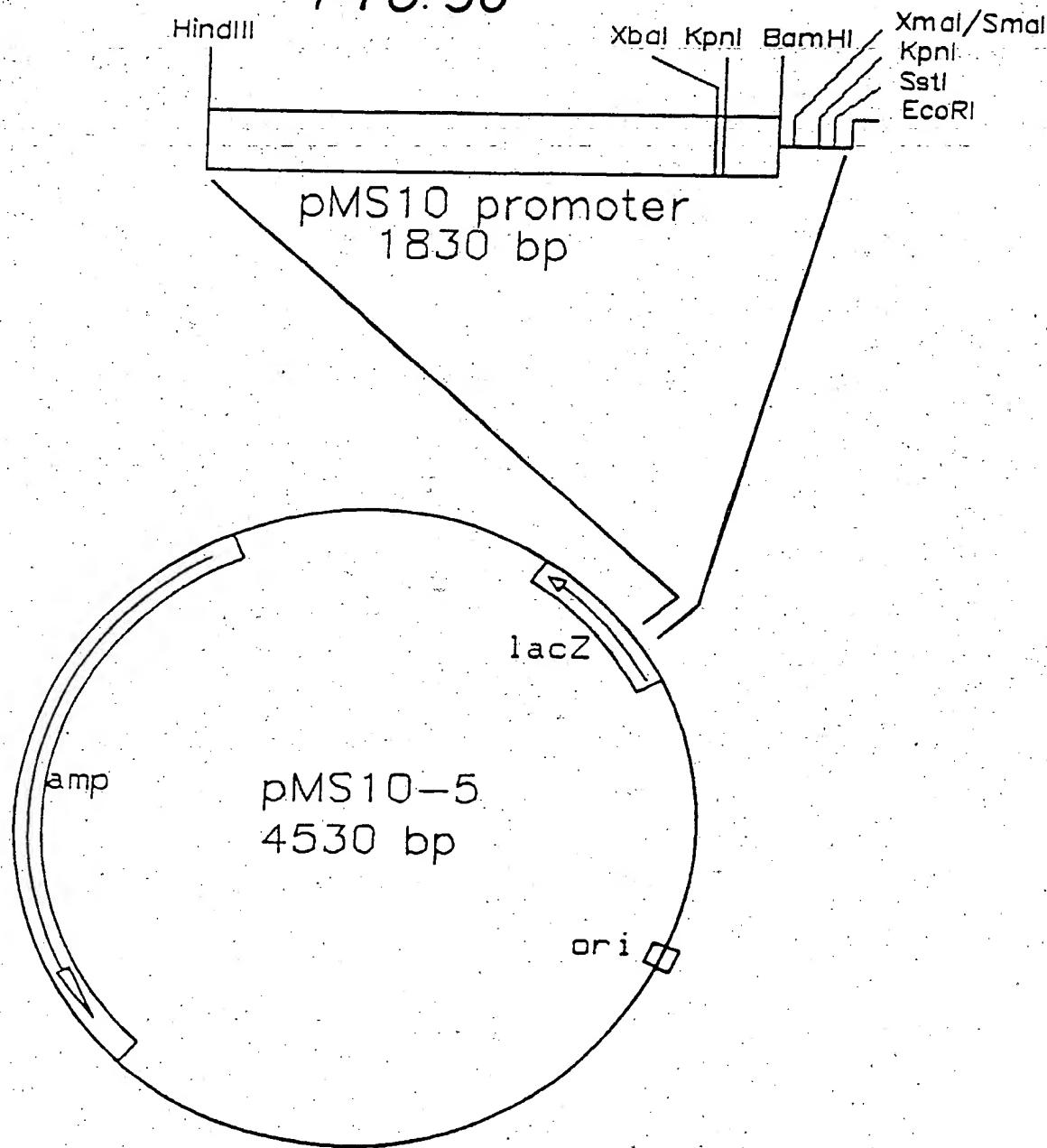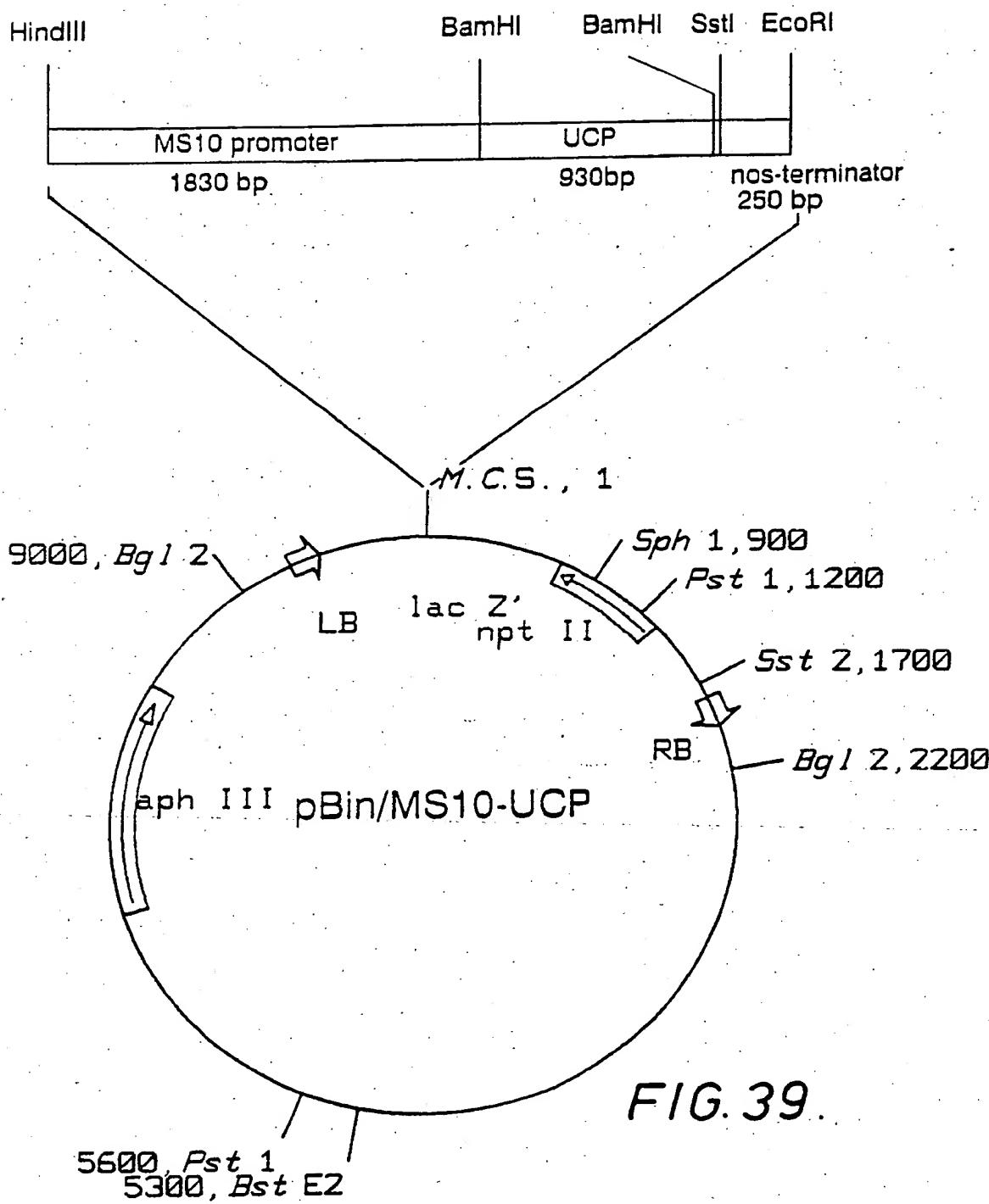
FIG. 38

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FIG. 39.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5: C 12 N 15/82, 5/10, A 01 H 5/00, // C 12 N 15/54, 15/12, 15/55, 15/29

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^5	C 12 N, A 01 H

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X	EP, A, 0329308 (PALADIN HYBRIDS) 23 August 1989 see page 6, lines 14-45	1
P,A		2-30
P,X	EP, A, 0332104 (CIBA-GEIGY) 13 September 1989 see page 17, lines 34-40	1
P,A		2-30
P,A	WO, A, 89/10396 (PLANT GENETIC SYSTEMS) 2 November 1989 see the whole document	1-30

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
8th May 1990	- 5. 06. 90

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MISS T. TAZELAAR

Form PCT/ISA/210 (second sheet) (January 1985)

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 28/05/90
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0329308	23-08-89	AU-A-	2963289	03-08-89
EP-A- 0332104	13-09-89	AU-A-	3108089	14-09-89
WO-A- 8910396	02-11-89	AU-A-	3537189	24-11-89
		EP-A-	0344029	29-11-89

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